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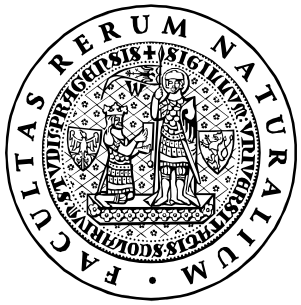

DISSERTATION THESIS

Vztah mezi genetickými polymorfismy DNA reparačních genů a jejich expresí u zdravé populace (s výhledem na stanovení u onkologických pacientů).

The relationship between genetic polymorphisms in DNA repair genes and their expression in healthy population (with the prospect of analysis in oncology patients)

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ABSTRACT

DNA damage response is a complex system responsible for protection of a cell against internal and external DNA damaging agents and in maintaining genome integrity. Many of genes participating in DNA damage response pathways are polymorphic. Genetic polymorphisms in coding and regulatory regions may have impact on the function of proteins encoded by the genes. Phenotypic effect of single nucleotide polymorphisms (SNPs) is subject of investigation in connection with the ability of a cell to manage genotoxic stress and subsequently, in relation to cancer susceptibility.

The aim of this thesis was to evaluate the association between SNPs in DNA repair genes (*hOGG1, XRCC1, XPC*) and cell cycle genes (*TP53, p21*^{CDKN1A}*, BCL2* and *BAX*) and their mRNA expression in peripheral blood lymphocytes from individuals occupationally exposed to styrene and control individuals. The aim was extended to analyses of relationships between mRNA expression levels of the above-mentioned genes and markers of exposure to styrene (concentration of styrene in blood and in air), markers of DNA damage (single strand breaks – SSBs, and endonuclease III specific sites – Endo III sites) and the base excision repair (BER) capacity, by means of γ-irradiation specific DNA repair rates and oxidative repair.

Study on the group of healthy individuals revealed that the γ-irradiation specific DNA repair rates were decreased in individuals bearing variant allele in *XRCC1* Arg399Gln; and the individuals with wild type genotypes *hOGG1* Ser326Cys had nearly 2-fold higher oxidative repair capacity. These results suggest a relationship between BER capacity and SNPs in DNA repair genes.

mRNA expression of *hOGG1, XRCC1, XPC* positively correlated with both markers of DNA damage, SSBs and Endo III sites. In the whole study population, negative correlations were found between mRNA expression of *TP53*, *BCL2*, *BAX*, and external and internal markers of styrene exposure. *p21*^{CDKN1A} mRNA expression significantly increased with increasing styrene exposure as well as with γ-irradiation specific DNA repair rates. Positive correlation between markers of DNA damage and mRNA expressions in *TP53* and *BCL2* was disclosed. Levels of SSBs and Endo III sites decreased with increasing *p21*^{CDKN1A} mRNA expression. However, our results did not

reveal any association between SNPs and mRNA expression levels of any of the studied genes. Furthermore, no induction of mRNA expression of DNA repair genes was observed with respect to styrene exposure.

In conclusion, DNA repair phenotype was shown to be modulated by genetic variability in DNA repair genes, and by occupational exposure to styrene. The occupational exposure had no influence on DNA repair genes mRNA expression, but seems to have effect on expression of genes involved in cell cycle control, as measured in lymphocytes. The four manuscripts constituting this thesis provide unique investigation of possible linkage between phenotypic effect of DNA repair, and mRNA expression of DNA repair and cell cycle genes in response to styrene exposure on human population.

ABSTRAKT

Odpověď na poškození DNA je komplexní systém, odpovědný za ochranu buňky proti vnitřním a vnějším vlivům poškozujícím DNA a za udržování integrity genomu. Mnoho genů, které se účastní signálních drah reagujících na poškození DNA, je polymorfních. Genetické polymorfismy v kódujících a regulačních oblastech mohou mít vliv na funkci proteinů, které tyto geny kódují. Fenotypový efekt jednonukleotidových polymorfismů (SNPs), je předmětem zkoumání v souvislosti se schopností buňky zvládat genotoxický stres a následně ve vztahu k riziku vzniku onkologického onemocnění.

Cílem této práce bylo zhodnotit vztah mezi SNPs v genech zodpovědných za opravy poškození DNA (*hOGG1, XRCC1, XPC*) a genech buněčného cyklu (*TP53, p21*^{CDKN1A}, *BCL2* a *BAX*) a expresí na úrovni mRNA v lymfocytech periferní krve u subjektů exponovaných styrenu v pracovním prostředí a u kontrolních subjektů. Cíl byl rozšířen na analýzu vztahu mezi expresí uvedených genů na úrovni mRNA a markery expozice styrenu (koncentrace styrenu v krvi a ve vzduchu), markery poškození DNA (jednořetězcové zlomy – SSBs; místa specifická pro endonukleázu III - Endo III místa) a kapacitou bázové excizní opravy (BER) měřené pomocí míry opravy poškození DNA způsobeného γ-zářením a schopností opravovat oxidativní poškození.

Studie na skupině zdravých jedinců odhalila, že míra opravy poškození DNA způsobeného γ-zářením byla snížená u jedinců s variantní alelou u *XRCC1* Arg399Gln; a jedinci s divokým typem genotypu *hOGG1* Ser326Cys měli téměř dvakrát vyšší kapacitu opravovat oxidativní poškození. Tyto výsledky naznačují, že může existovat vztah mezi kapacitou BER a SNPs v genech zodpovědných za opravu DNA.

Exprese na úrovni mRNA u genů *hOGG1, XRCC1, XPC* pozitivně korelovala s markery poškození DNA (SSBs a Endo III místa). Ukázalo se, že v celé studované populaci existují negativní korelace mezi expresí na úrovni mRNA genů *TP53*, *BCL2*, *BAX*, a externími a interními markery expozice styrenu. Exprese genu *p21*^{CDKN1A} na úrovni *mRNA* signifikantně stoupala se zvyšující se expozicí styrenu a pozitivně korelovala s mírou opravy poškození DNA způsobeného γ-zářením. Zjistili jsme také pozitivní korelaci mezi markery poškození DNA a expresí na úrovni mRNA u *TP53* a *BCL2*. Hladina SSBs a Endo III míst klesala se zvyšující se expresí *p21*^{CDKN1A} na úrovni mRNA.

Získané výsledky však neprokázaly vliv SNPs na expresi studovaných genů na úrovni mRNA. Kromě toho, indukce exprese genů DNA opravy na úrovni mRNA v odpovědi na expozici styrenu nebyla potvrzena.

Závěrem lze konstatovat, že fenotyp opravy DNA je modulován genetickou variabilitou v genech opravy DNA a expozicí styrenu v pracovním prostředí. Pracovní expozice nemá vliv na expresi genů opravy DNA, ale zdá se, že má vliv na expresi genů, které se podílejí na řízení buněčného cyklu, jak dokládají naše měření na lymfocytech. Čtyři publikace tvořící tuto práci poskytují jedinečné šetření možné spojitosti mezi fenotypovým účinkem opravy DNA a expresí genů DNA opravy a genů buněčného cyklu na úrovni mRNA v odpovědi na expozici styrenu u lidské populace.

LIST OF MANUSCRIPTS

This thesis is based on the following publications, which will be referred to by their Roman Numerals:

I
Vodicka P., Stetina R., Polakova V., Tulupova E., Naccarati A., Vodickova L., Kumar R., **Hanova M**., Pardini B., Slyskova J., Musak L., De Palma G., Soucek P., Hemminki K. (2007) Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects. *Carcinogenesis*. 28(3):657-6

II
Manini P., De Palma G., Andreoli R., Marczynski B., **Hanova M**., Mozzoni P., Naccarati A., Vodickova L., Hlavac P., Mutti A., Vodicka P. (2009) Biomarkers of nucleic acid oxidation, polymorphism in, and expression of *hOGG1* gene in styrene-exposed workers. *Toxicol Lett*. 8;190(1):41-7.

III
Hanova M., Stetina R., Vodickova L., Vaclavikova R., Hlavac P., Smerhovsky Z., Naccarati A., Polakova V., Soucek P., Kuricova M., Manini P., Kumar R., Hemminki K., Vodicka P. (2010) Modulation of DNA repair capacity and mRNA expression levels of *XRCC1, hOGG1* and *XPC* genes in styrene-exposed workers. *Toxicology and Applied Pharmacology* 248 (2010) 194–200.

IV
Hanova M., Vodickova L., Vaclavikova R., Smerhovsky Z., Stetina R., Hlavac P., Naccarati A., Slyskova J., Polakova V., Soucek P., Kumar R., Hemminki K., Vodicka P. (2011) DNA damage, DNA repair rates and mRNA expression levels of cell cycle genes (*TP53, p21*^{CDKN1A}*, BCL2* and *BAX*) with respect to occupational exposure to styrene. *Carcinogenesis*. Jan;32(1):74-9

ABBREVIATIONS

Arg	Arginine
AP	apurinic/apyrimidinic
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
B2M	Beta-2-Microglobulin
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma
BER	Base-excision repair
bp	base pair
Chk2	Chk2 checkpoint homolog
Cys	Cysteine
Da	Daltons
EndoIII sites	SSBs Endonuclease III sites
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gln	Glutamine
Gy	gray unit
IARC	International agency for research on cancer
hOGG1	8-hydroxyguanine DNA glycosylase
NER	nucleotide excision repair
p21	Cyclin-dependent kinase inhibitor 1A
PARP1	poly (ADP-ribose) polymerase 1
PCNA	Proliferating cell nuclear antigen
PBL	Peripheral blood lymphocytes
PPIA	Peptidylprolyl isomerase A (cyclophilin A)
qPCR	Quantitative PCR
SD	Standard deviation
Ser	Serine
SNP	single nucleotide polymorphism
SSBs	Single-strand breaks
TP53	Tumor protein p53
XPC	Xeroderma pigmentosum, complementation group C
XRCC1	X-ray repair cross-complementing protein 1
γ -irradiation	Gamma irradiation

1. INTRODUCTION

DNA is being continually damaged by a variety of chemical and physical agents and therefore all cells inevitably possess spectra of mechanisms for DNA repair. The ability to repair DNA damage is crucial for preventing loss of vital genetic information and for maintaining genome integrity. Eukaryotic DNA repair pathways are highly conserved from yeast to humans. In general, cells respond to DNA damage by activating checkpoint pathways that delay the progression through the cell cycle, promote DNA repair or induce cell death. The whole repair cascade depends on (but is not limited to): a type of genotoxic effect, a cell type, and a cell stage (proliferating, quiescent or differentiated cells). The whole process may result in many different outcomes such as repair of the damage, fixation of a mutation, initiation of a disease, initiation of carcinogenesis or cell death. Managing DNA damage by the cell and by the whole organism is of great importance in their survival and evolution.

1.1 DNA damage

Biological organisms are in their environment exposed to a number of physical and chemical factors having capacity to induce DNA damage. DNA damage may be induced spontaneously as a result of the cell metabolism or by environmental agents. Spontaneous alterations and lesions introduced as a consequence of cell metabolisms include spontaneous deaminations and loss of bases due to spontaneous hydrolysis, mismatches resulting from errors made by DNA polymerases during DNA replication or oxidative damage caused by reactive oxygen species etc.

Among genotoxic agents from external environment, it might be ionising radiation that is ubiquitous in the environment and has the ability to cause strand breaks, and DNA base damage indirectly through formation of free radicals. UV radiation causes two classes of DNA lesions: cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts. Both of these lesions distort DNA's structure, introducing bends or kinks and thereby impeding transcription and replication (Clancy, 2008).

Group of chemical agents include environmental carcinogens and industrial chemicals. Among them we shall mention monofunctional and bifunctional alkylating agents (methylnitrosourea; methyl-methansulphonate); crosslinking agents (Mitomycin, cisplatin, HNO_2), psoralens, chemicals metabolically activated to electrophilic reactants;

aromatic amines; polycyclic aromatic hydrocarbons; and aflatoxins (Report on carcinogens, 12th edition, NTP, 2011).

One of the most extensively used industrial chemicals in plastics industries for the production of various plastics and polyester resins is styrene. The compound is also present in small quantities in food; tobacco smoke and engine exhaust (IARC 2002). IARC classifies styrene as a possible human carcinogen (group 2B) and its principal metabolite, styrene-7,8-oxide, as a probable human carcinogen (group 2A, IARC 1994). In the Report on carcinogens, styrene was classified within the group "reasonably anticipated to be human carcinogens", based on limited evidence of carcinogenicity from studies in humans, sufficient evidence of carcinogenicity from studies in experimental animals, and supporting data on mechanisms of carcinogenesis (Report on carcinogens, 12th edition, 2011 NTP). Genotoxic effects of styrene and styrene-7,8-oxide have comprehensively been reviewed (Vodicka et al., 2006). It was postulated that the genotoxic effects are mainly associated with styrene biotransformation to styrene-7,8-oxide.

DNA damage caused by the above mentioned agents is processed on the cellular level. Cells possess effective mechanisms of DNA damage response to deal with genotoxic insults.

1.2 DNA damage response

DNA damage response (DDR) is a sophisticated system of pathways that plays a substantial role in the maintenance of genomic integrity. The proper response of a cell to DNA damage caused by endogenous agents and exogenous environmental contaminants is ensured through the cooperation between sensors, transducers and effectors of DDR (as illustrated in Fig 1.). DDR pathways includes detection of DNA lesions, signalling their presence and promoting their repair (Rouse and Jackson, 2002; Harrison and Haber, 2006; Harper and Elledge, 2007; Garner and Constanzo, 2009; Jackson and Bartek, 2009). The DDR machinery comprises critical proteins involved in cell cycle regulation, histone modifications, and DNA repair (Powell and Bindra, 2009; Harper and Elledge, 2007).

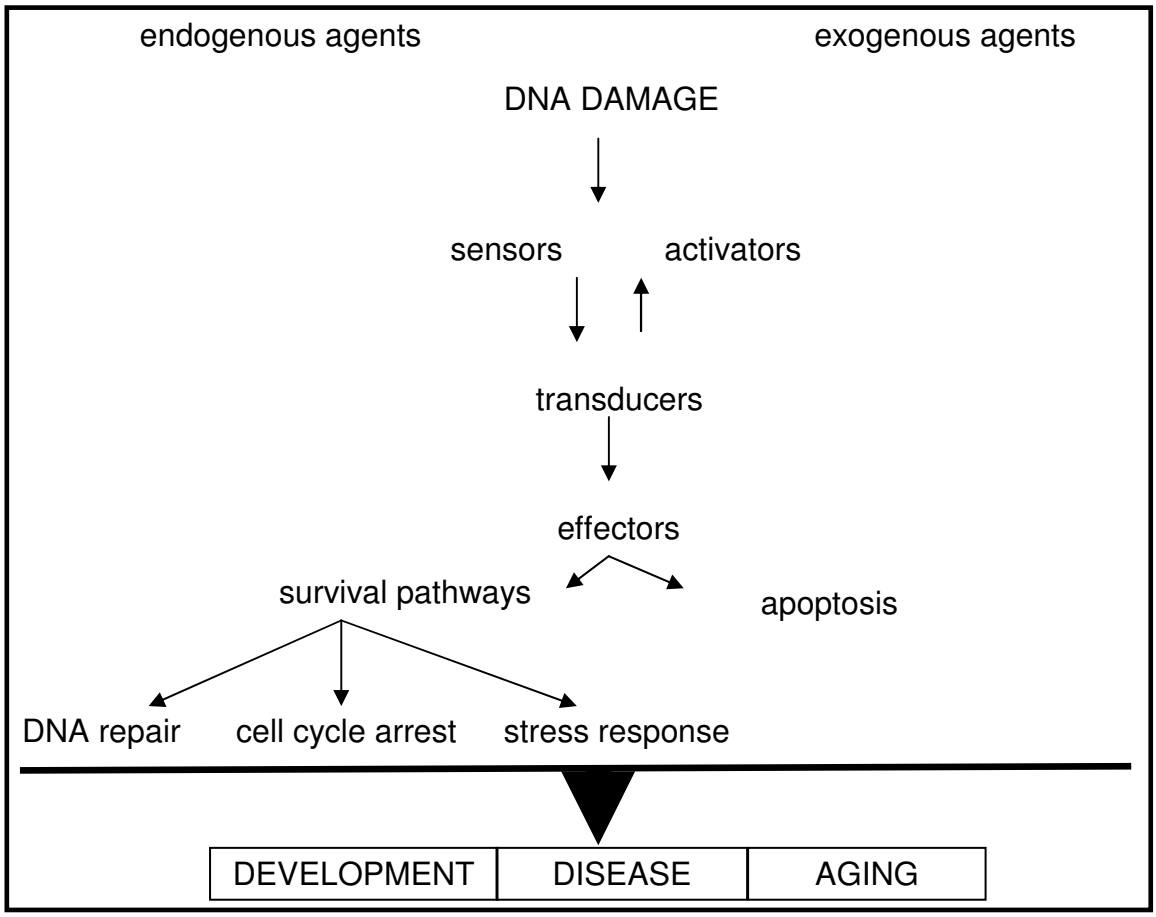

Figure 1. Biochemical and Physiological Consequences of the DNA Damage Response (modified from Harper and Elledge, 2007)

The key protein kinases responsible for mediating DNA damage are ATM and ATR. They act either together or separately in response to specific DNA damage and stalled replication. Both ATM and ATR are able to recognise double or single strand breaks and they act as recruiters of additional substrates and as scaffolds for assembling multi-protein complexes during mediation of DNA repair.

ATM and ATR regulation network comprises DNA repair, regulation of gene expression, RNA processing and chromatin organisation. They not only influence the downstream processes as a result of DNA damage, but the DDR signalling is multi-directional. It has been shown that ATM activates MRN (MRE11–RAD50–NBS1) complex and MRN complex stimulates the kinase activity of ATM toward its substrates TP53, Chk2, and histone H2AX (Lee and Paull, 2004).

Complete functional loss of *ATM* is a cause of the disease ataxia telangiectasia, a genomic instability syndrome that affects the nervous, immune and reproductive systems, and ultimately leads to cancer predisposition (Shiloh 2001).

Additional factor influencing DDR is modification of histone proteins (histone code). These modifications involve phosphorylation, ubiquitilation, sumoylation, acetylation and methylation. The histone code has an impact on DNA damage detection and repair, and influences chromatin accessibility to repair factors. For instance, following UV exposure histone acetylation takes place and it is believed to facilitate NER process (Escargueil et al., 2008).

DNA damage signalling enhances repair by inducing DNA repair proteins transcriptionally or post-transcriptionally; by recruiting repair factors to the damage; and by activating DNA repair proteins by modulating their phosphorylation, acetylation, ubiquitylation or sumoylation. Recent investigations suggest interplay between multiple protein modifications that combine to propagate the DNA damage signal to elicit cell cycle arrest, DNA repair, apoptosis and senescence (Huen and Chen, 2008; Pinder et al., 2013). For instance XRCC1 phosphorylation by casein kinase 2 is required for its stability and efficient DNA base excision repair (Parsons et al., 2010). *In vitro* study showed that TP53 stimulates BER by direct interactions with APE and polymerase β. TP53 stabilises the interaction between the polymerase and abasic DNA (Zhou et al., 2001).

DNA repair pathways may have different dynamics depending on cell type, developmental stage or physiological state of the cell(s), some are down-regulated upon cell differentiation in non dividing cells, comparing to DNA repair in proliferating cells. It was shown that quiescent and proliferating hematopoietic stem cells are equally radio-protected but use different types of DNA repair mechanisms in response to ionising radiation. Proliferating hematopoietic stem cells displayed an attenuated TP53-mediated response including limited induction of $p21^{CDKN1A}$ expression (Mohrin et al., 2010).

Downstream of the DNA damage signalling are the DNA repair pathways themselves. According to the specificity of DNA damage, distinct repair mechanisms were identified. The list of possible responses to DNA damage is stated in Table 1. Nevertheless, the complex repair process is influenced by post-translational modifications, interlocking signalling and repair pathways, overlapping substrate specifications of the repair processes and regulation of assembly of replication complexes. (Harper and Elledge, 2007; Jackson and Bartek, 2009; Milanowska et al., 2011).

Table 1. Biological responses to DNA damage modified from Friedberg, DNA repair and mutagenesis, 2006.

Biological response to DNA damage
Reversal of base damage
Excision of damaged, mispaired, or incorrect bases Base excision repair Nucleotide excision repair Transcription coupled nucleotide excision repair Alternative excision repair Mismatch repair
Strand break repair Single strand break repair Double strand break repair
Tolerance of base damage Translesion DNA synthesis Postreplicative gap filing Replication for progression
Cell cycle checkpoint activation
Apoptosis

1.3 Excision repair pathways and their implications in maintaining human genomic integrity

Base excision repair (BER) target mainly oxidative base damage, alkylation, deamination, sites of base loss, and single strand breaks. The insulting factors are, ionising radation, xenobiotic chemicals, or cellular metabolites. A damage site is recognised by monofunctional and bifunctional DNA glycosylases that catalyse hydrolysis of *N*-glycosidic bond of the damaged deoxynucleoside, and AP site is created. AP endonucleases hydrolyse the phosphodiester bond immedately 5' to the AP site. AP lyases (bifunctional DNA glycosylases) are also capable of cleaving AP sites but rather via β-elimination (elimination of its 3' phosphate) (Zharkov 2008; Fromme and Verdine 2004). The repair synthesis step in BER pathway can proceed in two different sub-pathways: short patch BER and long patch BER. During the short patch BER single dNMP is incorporated into DNA, and the nick is sealed by a DNA ligase. Long patch

repair is processed by PCNA, FEN-1 (a flap endonuclease) and DNA polymerases ε/δ. More extensive patch (2-20 dNMP) displaces stretch of old DNA into a flap structure which is then processed by FEN-1. The ligation is processed by ligase I and III, and DNA is covalently resealed. DNA ligase III interacts with XRCC1, polymerase β and PARP1, whereas DNA ligase I with PCNA.

Long patch repair is considered to be important for replication associated repair of base damage while by short patch way the entire altered nucleoside is removed and repair polymerisation can proceed directly (Christmann et al., 2003).

This work is focused on two genes from BER pathway: *hOGG1* and *XRCC1*.

hOGG1 belongs to a diverse group of DNA glycosylases related to endonuclease III (Nth superfamily); it is bifunctional and specific for oxidised purines (Zharkov 2008; Mirbahai et al., 2010).

XRCC1 is non-enzymatic scaffolding protein that stabilises the ligase; two BRCT domains in this protein are likely to mediate its interaction with ligase IIIα and PARP1 (Mani et al., 2004; Mani et al., 2007; Nazarkina et al., 2007). Binding of XRCC1 to polymerase β and ligase III is functionally important. Moreover XRCC1 stimulates the repair activity of hOGG1 on DNA containing 8-oxoguanine (Fromme and Verdine 2004).

The nucleotide excision repair (NER) has wide substrate specificity and ability to recognise and repair a large number of structurally unrelated lesions such as bulky DNA adducts (benzo[a]pyrene guanine adduct, acetylaminofluorene-guanine adduct, cisplatin-d (GpG) diadduct) and lesions induced by UV light (cyclobutane pyriminide dimer, (6-4) photoproducts). Two distinct pathways are recognized in NER: global genome repair and transcription coupled repair.

A key aspect of NER is that the damage is excised as a 22–30-base oligonucleotide, producing single-stranded DNA that is processed by DNA polymerases and associated factors before ligation ensues (Huang et al., 1992). Multiple proteins take part on DNA damage recognition, excision, polymerisation and ligation (Table 2).

Table 2. Human genes involved in nucleotide excision repair (modified from Friedberg, 2001)

Human gene / protein	**Function in NER**
XPC/XPC	Involved in damage recognition. Not required for transcription coupled NER.
RAD23B *(hHR23B)* / HRAD23B	Binds to XPC. Involved in damage recognition. No human mutants known
RAD23A (*hHR23A*) / HRAD23A	Can substitute for HRAD23B, no human mutants known
CENT2 / centrin2 (or caltacin 1)	Stabilizes XPC in the presence of HRAD23. No human mutants known
XPA / XPA	Involved in damage recognition. Represented in human XP.
RPA1 / RFA1	Subunit of trimeric RFA complex. Involved in damage recognition. No human mutants known
RPA2 / RFA2	Subunit of RFA complex. Involved in damage recognition. No human mutants known
RPA3 / RFA3	Subunit of RFA complex. Involved in damage recognition. No human mutants known
XPB / XPB	Subunit of core TFIIH complex. 3′→5’ DNA helicase. Promotes bubble formation. Represented in human Xeroderma pigmentosum (XP)/Cockayne syndrome
XPD / XPD	Subunit of core TFIIH. 5′→3’ DNA helicase Promotes bubble formation. Represented in human XP, XP/CS syndrome and Trichothiodystrophy (TTD).
GTF2H1 / p62	Core TFIIH subunit. Promotes bubble formation. No human mutants known
GTF2H2 / p44	Core TFIIH subunit. Promotes bubble formation. No human mutants known
GTF2H3 / p34	Core TFIIH subunit. Promotes bubble formation. No human mutants known
GTF2H4 / p52	Core TFIIH subunit. Promotes bubble formation. No human mutants known
XPG / XPG	3’ DNA-structure-specific endonuclease.Required for bimodal incision. Represented in human XP and XP/CS syndrome.
ERCC1 / ERCC1	5’ DNA -structure-specific endonuclease with XPF Required for bimodal incision. No human mutants known.
XPF / XPF	5’ DNA -structure-specific endonuclease with ERCC1. Required for bimodal incision. Represented in human XP.
DDB1 / DDB1	Forms a complex with DDB2. Complex defective in individuals with XP-E.
DDB2 / DDB2	Forms a complex with DDB1. Complex defective in individuals with XP-E
CSA / CSA	Required for TCNER. Represented in human Cockayne syndrome (CS)
CSB / CSB	Required for TCNER. Represented in human Cockayne syndrome (CS)
XAB2 / XAB2	Interacts with XPA, CSA and CSB. Involved in transcription coupled NER. No human mutants known.

Global genome repair can detect and remove lesions throughout the genome. The process starts by damaged base recognition, followed by bimodal incision of DNA and the

excision of an oligonucleotide fragment. DNA damage is recognised by XPC protein. XPC couples with XPA and RPA proteins. RPA (replication protein A) is itself a complex of three proteins. Further hHRAD23B binds to XPC. *In vitro* studies showed NER can proceed without hHRAD23B however the efficiency was improved in its presence (Sugasawa et al., 1996). The XPC/hHRAD23B complex is being stabilised by centrin 2/caltractin. This association implies the existence of regulatory relationship between NER and cell division. XPA and RPA are thought to bind to DNA after binding of XPC/hHRAD23B. Another protein of the NER machinery is transcriptional factor II H (TFIIH), responsible for unwinding. Endonuclease XPG cuts damaged DNA strand 3' to a site of damage and XPF-ERCC1, a heterodimeric protein, cuts damaged DNA strand 5' to the site. Re-synthesis occurs by polymerases δ and ε and ligation by ligase I. (Friedberg 2001; Christmann et al., 2003)

Transcription coupled repair ensures faster repair of many lesions when located on the transcribed strand of actively transcribed genes. Damage recognition in this type of repair is recognised by arrested RNA polymerase II transcription machinery. CSA and CSB are recruited to the site. RNA polymerase II is then removed from the site providing thereby the access to endonucleases XPF-ERCC1 and XPG. Further step in the process is ensured by polymerases δ and ε and ligase I and transcription is restarted (Friedberg 2001; Christmann et al., 2003).

Each DNA repair pathway deals with specific types of lesion except for some overlaps. NER plays a back up role for BER by removing non bulky DNA lesions such as thymine glycols and 8-oxoguanine adducts at slow but physiologically relevant rate (Sancarn and Readorn, 2004).

Connections between BER and NER have been documented. The evidence of *in vitro* functional interaction between *XPC* and *TDG* (thymine DNA glycosylase) was described, wherein *TDG* is involved in the initiation of BER and *XPC-HR23B* complex functions as a damage recognition factor for global genome NER (Shimizu et al., 2003). Also, in the absence of *XPC* a decrease in the repair rate of 8-oxoguanine was observed. It has been demonstrated that *XPC–HR23B* complex acts as cofactor in BER of 8-oxoguanine, by stimulating the activity of its specific DNA glycosylase hOGG1. (D'Errico et al., 2006).

The expression levels of BER and NER repair genes may change in response to genotoxic stress, and only limited number of *in vivo* studies are available. Concerning genes involved in BER, expression of the oxidative base excision repair enzymes has been studied in TK6 human lymphoblastoid cells after low doses of ionizing radiation. No

induction at the transcriptional level of any of the base excision repair genes *NTH1 (NTHL1), OGG1, NEIL1, NEIL2, NEIL3, APE1, POLB*, or accessory protein genes, *LIG3, XRCC1 or XPG*, was found at gamma-irradiation doses ranging from 0.01 to 2 Gy in a 24-h period. Only a dose-dependent induction of $p21^{CDKN1A}$ mRNA levels was observed in TK6 cells in the dose range between 0.5 and 2.0 Gy (Inoue et al., 2004).

Expression levels of genes involved in NER have so far been addressed mainly in relation to the malignant diseases. *XPC* mRNA levels have been studied in peripheral blood and squamous cell carcinoma of the head and neck tumour tissue samples. Significantly lower expression levels of *XPC* have been found in cases than in controls (Yang et al., 2005).

Expressions of *CSA, CSB, XPC, hHR23B, XPA, XPB, ERCC1* and *TP53* genes have been studied in hepatocellular carcinoma (HCC) cases, matching non-tumour tissue, and in normal liver biopsies from controls. The findings strongly suggest that over-expression of two key genes involved in the early steps of the NER process, *ERCC1* and *XPC*, is associated with liver fibrogenesis and cancer. *CSA, CSB, XPC, hHR23B, XPA, XPB, ERCC1* and *TP53* were frequently over-expressed in HCC compared to matched non tumour tissues (Fautrel et al., 2005).

1.4 Cell cycle and apoptosis

When the cell undergoes the genotoxic stress, changes in gene expression of genes involved in DDR occur as a consequence. One of the relevant genes is *TP53* that mediates apoptosis or cell cycle arrest and links DNA repair pathways with cell cycle (Agarwal et al., 1995; Zhou et al., 2001). Following DNA damage the TP53 protein rapidly accumulates and becomes activated. This activation represents a complex process, as summarized e.g. by Appella et al. 2001. Activation of TP53 results either in the cell cycle arrest in G1 allowing the repair to take place or in apoptosis, if DNA damage is too extensive to be repaired (Ko and Prives, 1996; May and May 1999). The critical effector of the TP53 is the cyclin-dependent kinase inhibitor $p21^{CDKN1A}$ protein. $p21^{CDKN1A}$ mediates G1 arrest in response to DNA damage. It associates with a cyclin/cdk/PCNA complex and inhibits kinase activity, thus blocking cell cycle progression into S phase (Gartel and Tyner 1999; Meek 2004).

The BCL2 family proteins are involved in the control of apoptosis and can either function as inhibitors (e.g. BCL2, BCL XL, BAG) or promoters (BAX, BCL XS, BAD, BAK) of cell death (Burger et al 1998). Some of these proteins physically interact with each other

and form homo- and heterodimers. It was proposed that BAX homodimers promote apoptosis and that the BAX mediated cell death is counteracted by BCL2/BAX heterodimerisation (Andreeff et al., 1999). TP53 can positively regulate *BAX* gene expression and is involved in negative regulation of *BCL2* gene expression (Lotem and Sachs, 1999). Therefore TP53 status may determine the susceptibility of cells to apoptotic stimuli by modulation of the BCL2/BAX complex.

The cell cycle machinery as a part of DDR plays an important role in response to genotoxic insult/exposure. The mechanisms of the effect of styrene-7,8-oxide on the expression of *TP53, p21*^{CDKN1A}*, BCL2* and *BAX* genes, in human lymphocytes, was investigated at the beginning of the millennium. A high inter-individual variation in the expression of the genes was observed. The results obtained for the four genes suggest that exposure to high dose of styrene-7,8-oxide may induce a delay in the cell cycle (to enable repair systems to act on the genotoxic damage produced), rather than driving cells towards apoptosis. This conclusion was further supported by cytokinesis block assay and DNA fragmentation assay (Laffon et al., 2001). Furthermore, an effect of styrene on gene expression profiles of human mononuclear cord blood cells as well as styrene modulation of apoptosis and BCL2 related protein expression in *in vitro* study was observed. Modified expression of BAX, BCL2, c-jun, c-fos, and raf-1 proteins was revealed in the human cord blood cells after direct exposure to styrene, whereas TP53 expression did not change (Diodovich et al., 2004).

1.5 DNA repair polymorphisms and their phenotypic effect

To assess human genotoxic exposure and early effects of genotoxic carcinogens, number of biological biomarkers has been used in epidemiological studies. Various biomarkers of internal exposure, susceptibility and biological effect analysed in peripheral blood lymphocytes became a criterion for estimation of a level of exposure to xenobiotics and to predict possible cancer risk and development of long-term diseases. Among these biomarkers are chromosomal aberrations; DNA breaks analysed by comet assay; DNA repair capacity tests, and genetic polymorphisms (Collins, 1996; Vodicka et al., 2003).

It is assumed that some SNPs may result in alteration of the protein function. Missence SNPs in DNA repair genes thus may have an impact on overall DNA repair capacity and ultimately may increase the risk of cancer, often via impaired genome integrity.

The mode of action how SNPs in genes encoding proteins that participate in DDR could modulate human response to genotoxic insult is of great interest. Various studies on styrene-exposed individuals and general populations showed associations between SNPs in genes involved in metabolism of xenobiotics and DNA repair, and intermediary molecular markers involved in the cascade of genotoxic events (Vodicka et al., 2004 a, b, c; Slyskova et al., 2007).

During the last decade several studies shown that SNPs in coding and regulatory sequences may result in subtle structural alterations in biotransformation and DNA repair enzymes, modulating cancer susceptibility (Naccarati et al., 2007; Theodoratou et al., 2012). In general, there were large amount of studies investigating the possible effect of SNPs on increased or decreased susceptibility to cancer. SNPs of various xenobiotic-metabolising enzymes, influencing the metabolic activation and detoxification of carcinogens, were associated with cancer risk (Norppa 2004; Skjelbred et al., 2011; Berwick and Vineis 2000).

Several epidemiological studies address the individual repair capacity as a parameter that may reflect the variability in ability to maintain genome integrity. Recent study on healthy population revealed high inter-individual variability in NER repair capacity and significant positive association of repair capacity with family history of cancer (Slyskova et al., 2011).

Intensive search has been underway for low penetrance genes that subtly influence DNA damage occurrence, cellular response to damage and DNA repair. A collective effect of low penetrance genes is thought to affect significantly individual cancer susceptibility (Shields and Harris 2000; Hemminki et al., 2006; Polakova et al., 2009; Pardini et al., 2008; Vodicka et al., 2001; Vodicka et al., 2003; Paz-Elizur et al., 2008). Recent results from genome-wide association studies of colorectal cancer susceptibility that assessed a set of SNPs in 157 DNA repair genes showed no evidence of association of individual SNP with the disease. However SNP set analysis revealed that variation around DNA repair loci does contribute to colorectal cancer risk. (Tomlinson et al., 2012).

Considering the genetic variability in DNA repair genes in particular, there is a great interest in characterizing known polymorphic sites for their functional outcome. The great tool of studying this issue is to measure DNA repair phenotype, by means of DNA repair capacity to recognise and remove experimentally-induced DNA damage. DNA repair capacity represents a complex marker comprising several factors such as gene variants, gene expression, stability of gene products and environmental factors.

2. AIMS

The aim of the study was to characterise healthy population occupationally exposed to styrene for distribution of polymorphic variants in DNA damage response genes and to investigate their relation to mRNA expression of these genes; and further to evaluate both SNPs and mRNA expression of the selected genes in context with markers of occupational exposure to styrene (concentration of styrene in blood and in the air), markers of DNA damage (DNA strand breaks and endonuclease III-specific sites) and finally, the BER phenotypes.

Specific aims of this Thesis are summarized in the following points:

1. Evaluation of the association between SNPs in selected DNA repair genes and their mRNA expression levels in control individuals and individuals occupationally exposed to styrene.

2. Evaluation of mRNA expression levels in selected DNA repair genes and genes of cell cycle control in styrene exposed and control individuals with respect to markers of occupational exposure to styrene.

3. Evaluation of mRNA expression levels in selected DNA repair genes and genes of cell cycle control in styrene exposed and control individuals with respect to DNA damage and phenotypic outcome of BER.

4. Overall evaluation of acquired data with respect to genotoxicity and molecular mechanisms of cell response to styrene exposure and DNA damage.

3. METHODS

3.1 Study population

Manuscript I:

The study was conducted on 244 healthy individuals (183 men and 61 women, mean age (±SD) 41.3 ± 11.3 years, 90 individuals were smokers and 154 non-smokers) employed in local administration, medical centres and various branches of plastic industry. The investigated population was recruited in the regions of western Slovakia and eastern Bohemia, which exhibit close similarities in socio-economical conditions. Confounding factors, like X-rays, medical drug treatments, dietary and lifestyle habits and possible exposure-related effects were recorded in detailed questionnaires and considered in the statistical analyses.

Manuscript II:

Sixty styrene-exposed workers employed in two plastics lamination plants in the same geographical area (mean age±SD was 37.6±11.1 years) and 50 unexposed control individuals (mean age±SD was 40.0±12.1 years) volunteered to participate in the study. Confounding factors, like X-rays, medical drug treatment, dietary and lifestyle were carefully controlled by detailed questionnaire. The study was conducted on healthy individuals and exclusion criteria comprised a recent exposure to X-rays, current drug use or viral infections experienced in the last 3 months.

Manuscripts III and IV:

The styrene-exposed group consisted of 71 workers employed in the hand lamination (with age of 38.6±12.2 years; mean±SD); the mean length of occupational exposure was 5.2 ± 4.0 years (mean ± SD). Fifty-one workers employed as mechanics in a local car plant represented the control group (40.0±12.0 years). The differences in the styrene exposure were reflected by the stratification of the studied group into three sub-groups according to the level of styrene concentration at workplace. In the control group styrene concentration was below the limit of detection. The exposed group was arbitrarily divided into those with low styrene exposure (below 50 mg/m^3) and with the high styrene exposure (above 50 mg/m^3).

3.2 SNP analyses

SNPs in DNA repair genes were determined by a PCR–RFLP based method. PCR products were generated using 50 ng of genomic DNA in 25 ml volume reactions containing 20 mM Tris–HCl, 50 mM KCl, 2.0 mM MgCl2, 0.3 mM each dNTP, 0.3 mM each primer and 0.2 U Taq DNA polymerase. The temperature conditions for PCR were as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s and final extension at 72°C for 5 min. The amplified fragments were digested with appropriate restriction endonucleases. The digested PCR products were resolved on 3% agarose gels containing ethidium bromide and visualized under UV light. (Manuscripts I and II).

3.3 DNA damage and DNA repair rates

DNA damage was measured as SSBs and more specifically as Endo-III sites. SSBs in PBL were measured by means of the alkaline version of the comet assay. Using this assay, alkali-labile sites may represent alkali-labile DNA adducts, oxidized bases, abasic sites, true DNA breaks as well as transient gaps appearing in the DNA during DNA repair. To determine abasic sites and oxopyrimidines more specifically, we measured on parallel slides Endo-III sensitive sites by incubating lysed nucleoids with endonuclease III enzyme, afterwards electrophoresis was carried out as for SSBs analysis (Manuscripts III and IV).

The oxidative DNA repair was analyzed as the capacity of extracts from PBL to repair 8-oxoguanines. Aliquots from lymphocyte extracts were pipetted on microscopic slides with HeLa cells mounted in agarose for comet assay. HeLa cells were pre-treated with photosensitizer Ro 19-8022 (Hoffmann-La Roche, Basel, Switzerland) and irradiated with fluorescent lamp to induce 8-oxoguanines in the DNA. HeLa cells with induced oxidative DNA damage were incubated with PBL extracts resulting in introduction of SSB at the place of 8-oxoguanines. Results represent an amount of SSBs created by removal of 8-oxoguanines by specific 8-oxoguanine glycosylase and are expressed as SSBs/10^9 Da (Manuscripts II, III and IV).

To determine DNA repair capacity for removal of γ-irradiation-induced SSBs, peripheral blood lymphocytes were embedded in agarose on microscope slides were irradiated on ice by 5 Gy of gamma rays. One of the two parallel slides was immediately processed for

the comet assay, whereas the other was first incubated in culture medium to allow the repair of DNA breaks induced by irradiation. The results (i.e. the amount of repaired SSBs) are calculated as the difference between the initial levels of SSBs, measured immediately after the irradiation, and the level of SSBs, detected after 45 min of incubation. The repaired DNA damage is expressed as SSBs/10^9 Da (Manuscripts II, III and IV).

3.4 Expression analyses

3.4.1 RNA isolation

PBLs were isolated from 10 ml of venous blood and total RNA was immediately isolated using TRIzol according to the procedure supplied by the manufacturer (Invitrogen, Paisley UK). RNA quantity and quality was assessed by UV-VIS spectrophotometry on Cary 300 (Varian Palo Alto, CA) and horizontal agarose gel electrophoresis.

cDNA was synthesized using 0.5 or 1 microgram of total RNA by help of RevertAidTM First strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania) with random hexamer primers.

3.4.2 qPCR

Manuscript II:

mRNA expressions of target gene *hOGG1* and reference gene *GAPDH* were determined by real time PCR in 7500 Real Time PCR System using TaqMan Universal master mix (catalogue no. 4364341) and TaqMan Gene Expression Assays Hs00213454_m1 /hOGG1/, and TaqMan Endogenous control Human GAPDH (4333764). All qPCR reagents were provided by Applied Biosystems (Foster City, CA, USA). As standards for absolute quantification of gene expression, bacterial plasmids pDONR221 containing coding sequences of *hOGG1* and *GAPDH* were used. Constructs were prepared by the GatewayTM cloning technology (Invitrogen, Paisley UK). The mRNA expression of *hOGG1* was normalized to the expression of *GAPDH* (TaqMan Endogenous control Human GAPDH 4333764) and expressed in arbitrary units.

Manuscripts III and IV:

mRNA expressions of target genes *XPC, XRCC1, hOGG1, TP53, p21*^{CDKN1A}*, BCL2* and *BAX* and reference genes *B2M, GAPDH* and *PPIA* were determined by real time PCR in

7500 Real Time PCR System using TaqMan Universal master mix (catalogue no. 4364341) and TaqMan Gene Expression Assays 4331182 (Hs00959834_m1/XRCC1/, Hs00213454_m1 /hOGG1/, Hs00190295_m1 /XPC/), Hs00153349_m1/TP53/, Hs00355782_m1/CDKN1A/, Hs00153350_m1/BCL2/, Hs00180269_m1/BAX/, Human PPIA Taqman Pre-developed Assay (4333763); TaqMan Endogenous control Human Beta-2-Microglobulin (4333766) and GAPDH (4333764)). All qPCR reagents were provided by Applied Biosystems (Foster City, CA, USA).

As standards for absolute quantification of gene expression, bacterial plasmids pDONR221 containing coding sequences of *XPC, XRCC1, hOGG1, BCL2* and *BAX*, *B2M, PPIA* and *GAPDH* were used. Constructs were prepared by the GatewayTM cloning technology (Invitrogen, Paisley UK). As standards for $p21^{CDKN1A}$, and *TP53* genes the PCR products were used.

All primers were designed using free software available at http://frodo.wi.mit.edu/primer3 and are stated in Table 3. cDNA from the samples was diluted 10-times, the expression level was determined as number of copies per µg of total RNA. The real time PCR was carried out in a final volume of 20 µl containing 5 µl of diluted cDNA. Cycling program was set at initial hold at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 sec., annealing and extension at 60°C for 1 min. Results were analyzed using the integrated 7500 System SDS Software version 1.3.1.

The expressions of *XPC, XRCC1, hOGG1, TP53,* $p21^{CDKN1A}$*, BCL2* and *BAX* were normalized to the normalization factor calculated from mRNA expression levels of reference genes *B2M, PPIA* and *GAPDH* using algorithm GENORM (http://medgen.ugent.be/~jvdesomp/genorm/). The cut off point for the gene expression stability measure M was set to 0.15.

Table 3. Primers for genes of interest (*XPC, XRCC1, hOGG1, TP53*, $p21^{CDKN1A}$, *BCL2* and *BAX*) and reference genes (*PPIA*, *B2M* and *GAPDH*)

Gene name	Primer	Sequence
XPC	Forward[a]	**ACCATATG**AATTTGCCCAGACAAGCAAC
	Reverse[b]	**AGTCGAC**TCCAATGAACCACTTCACCA
XRCC1	Forward[a]	**ACCATATG**GCTGGGGAGCAAGACTATGA
	Reverse[b]	**AGTCGAC**GCTGGGGGTCTTCTTTTCTT
hOGG1	Forward[a]	**ACCATATG**GAGCAAAGTCCTGCACACTG
	Reverse[b]	**AGTCGAC**TTCCTGAGATGAGCCTCCAC

TP53	Forward	GTGGAAGGAAATTTGCGTGT
	Reverse	TTTGGGTCTTTGAACCCTTG
p21^{CDKN1A}	Forward	GGAAGACCATGTGGACCTGT
	Reverse	TTCCTAAGAGTGCTGGGCAT
BCL2	Forward[a]	***ACCATATG***ATGTGTGTGGAGAGCGTCAA
	Reverse[b]	***AGTCGAC***TTTTCCATCCGTCTGCTCTT
BAX	Forward[a]	***ACCATATG***TTTGCTTCAGGGTTTCATCC
	Reverse[b]	***AGTCGAC***CTCAGCCCATCTTCTTCCAG
PPIA	Forward[a]	***ACCATATG***AGGGTTCCTGCTTTCACAGA
	Reverse[b]	***AGTCGAC***CCCAGTTGCTGCCTACATTT
B2M	Forward[a]	***ACCATATG***GTGCTCGCGCTACTCTCTCT
	Reverse[b]	***AGTCGAC***TCTCTGCTCCCCACCTCTAA
GAPDH	Forward[a]	***ACCATATG***CTCTGCTCCTCCTGTTCGAC
	Reverse[b]	***AGTCGAC***TTCTAGACGGCAGGTCAGGT

Restriction sites highlighted in **Bold.**
[a]Forward primer – restriction site for: *Nde*I
[b]Reverse primer – restriction site for: *Sal*I

3.5 Statistical analyses

Due to asymmetric distribution of the variables of interest, we employed the Kruskal-Wallis (K-W) nonparametric statistical test and Spearman correlation at bivariate level of analysis. When appropriate, the distributions of native values were transformed (logarithmic transformation or square root transformation) and parametric tests were applied to test the associations between the studied endpoints and explanatory variables. At this stage of analyses, we used the Pearson correlation analysis and the analysis of variance.

The simultaneous effects of explanatory variables on the studied endpoints were analyzed by means of linear multivariable regression. As independent predictors, we used the variables describing sex, age, smoking, and concentration of styrene in blood, duration of exposure in years and mRNA expression levels of studied genes. The adjusted R2 values and levels of statistical significances are reported for each model. All statistical analyses were carried out using SPSS v 16.0 (SPSS Inc., Chicago, IL, USA).

4. RESULTS

Manuscript I

Study "*Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects*" on the group of healthy individuals did assess the associations between SNPs in DNA repair genes and γ-irradiation-specific DNA repair rates and oxidative repair. The substantial finding in this study was that γ-irradiation-specific DNA repair rates were affected by SNPs in *XRCC1* Arg399Gln ($p<0.001$). Individuals bearing wild type genotypes in *XRCC1* Arg399Gln had significantly higher γ-irradiation-specific DNA repair rates comparing to variant genotypes ($p=0.0006$). Oxidative repair capacity was decreased in individuals with variant genotype in *hOGG1* Ser326Cys in comparison to wild type genotype ($p=0.008$). No other association between γ-irradiation-specific DNA repair rates and SNPs in genes involved either in NER (*XPD* Lys751Gln, *XPG* Asn1104His and *XPC* Lys939Gln) or DNA recombination repair (*XRCC3* Thr241Met and *NBS1* Glu185Gln) was shown.

Manuscript II

Study "*Biomarkers of nucleic acid oxidation, polymorphism in, and expression of, hOGG1 gene in styrene-exposed workers*" evaluated a modulating role of mRNA expression of *hOGG1* and *hOGG1* Ser326Cys polymorphism on oxidative DNA damage associated with styrene exposure. In the whole study group individuals with wild type genotype *hOGG1* Ser326 showed lower levels of 8-oxo-7,8-dihydroguanosine in lymphocytes compared to individuals with variant genotype ($p=0.008$). Furthermore, increased level of *hOGG1* mRNA expression was found in styrene exposed individuals compared to the controls ($p<0.0005$). In the study population *hOGG1* Ser326Cys polymorphism did not affect the levels of *hOGG1* mRNA expression.

Manuscript III

In study "*Does the occupational exposure to styrene modulate DNA repair rates and mRNA expression levels in XRCC1, hOGG1 and XPC genes?*", the analyses of associations between markers of styrene exposure, DNA damage, DNA repair capacity and mRNA expression levels were conducted.

The mRNA expression levels of *XRCC1*, *hOGG1* and *XPC* were significantly lower in the exposed groups as compared to the control group ($p<0.0001$ for each of the genes).

mRNA expression levels of *XRCC1*, *hOGG1* and *XPC*, based on the analysis of the whole study population, decreased with increasing styrene concentration in blood (R=-0.40, p=0.0001 for XRCC1, R=-0.66, p=0.0001 for hOGG1, and R=-0.36, p=0.0001 for XPC).

We observed that markers of DNA damage (SSBs and Endo III sites, which detect oxidised pyrimidines and apurinic/apyrimidinic sites,) measured in lymphocytes increased with the increasing mRNA expression levels of *XRCC1*, *hOGG1* and *XPC*. Only mRNA expression levels of *XPC* negatively correlated with γ-irradiation-specific DNA repair rates (R=-0.21, p=0.047). mRNA expression of *XRCC1* and *hOGG1* did not have any association to γ-irradiation-specific DNA repair rates.
mRNA expression levels of *XPC* moderately increased with smoking (R=0.27, p=0.041). No correlation between age and mRNA expression of any gene studied was revealed.
DNA damage and DNA repair rates (irradiation- as well as oxidative damage-induced) were also analysed in multivariate regression models. The dependent variables that were tested included concentration of styrene in blood, duration of exposure and mRNA expression of the investigated genes. The SSBs levels were significantly affected by *hOGG1* mRNA expression (p=0.017), Endo III sites were affected by *XRCC1* mRNA expression (p=0.001).

Manuscript IV

In study *"DNA damage, DNA repair rates and mRNA expression levels of cell cycle genes (TP53, $p21^{CDKN1A}$, BCL2 and BAX) with respect to occupational exposure to styrene"*, significant differences in mRNA expression levels of studied genes were found between the styrene exposed and control groups. mRNA expression levels of *TP53, BCL2* and *BAX* decreased with increasing concentration of styrene at workplace (R=-0.75, p<0.001; R=-0.65, p<0.001; R=-0.48, p<0.001, respectively) and styrene concentration in blood (R=-0.74, p<0.001; R=-0.62, p<0.001; R=-0.50, p<0.001, respectively).
On the contrary, $p21^{CDKN1A}$ mRNA expression levels increased with increasing concentrations of styrene at workplace and in blood (R=0.64, p<0.001 and R=0.40, p<0.001, respectively). All these correlations remained statistically significant after restricting the statistical analyses to exposed subjects only.

Positive correlations were found between SSBs and EndoIII sites and mRNA expression levels of both *TP53* (R=0.39, p<0.001; R=0.40, p<0.001, respectively) and *BCL2* (R=0.21, p=0.04; R=0.36, p=0.002, respectively).

However, levels of SSBs and EndoIII sites decreased with increasing $p21^{CDKN1A}$ mRNA expression levels (R=-0.51, p<0.001; R=-0.31, p=0.007, respectively). No association was found between mRNA expression levels of *BAX* and markers of DNA damage.

A positive association between mRNA expression levels of $p21^{CDKN1A}$ and γ-irradiation specific DNA repair (R=0.207, p=0.044) was found. No associations between mRNA expression levels of *BAX, BCL2* and *TP53* and γ-irradiation specific DNA repair were revealed.

5. DISCUSSION

Manuscript I

γ-irradiation specific DNA repair rates were significantly higher among individuals with the wild-type genotype in *XRCC1* Arg399Gln as compared to those with homozygous variant genotype. This result is in accordance with the results of the previous study where irradiation specific DNA repair rates were affected by the same SNP in *XRCC1* Arg399Gln and also by *XPC* Lys939Gln (Vodicka et al., 2004 b). Further study on styrene exposed population confirmed the effect of *XRCC1* Arg399Gln, where capacity to repair 8-oxoguanine was significantly lower in subjects with the variant genotype in *XRCC1* Arg399Gln (Slyskova et al., 2007). *XRCC1* has substantial role especially in BER and an observation of the decreased DNA repair capacity in individuals bearing XRCC1 399Gln is also supported by the cytogenetic challenge assay (Au et al., 2003), protein conservation analysis (Savas et al., 2004) and by increased irradiation sensitivity in lymphocytes from cancer free women (Hu et al., 2001). These data might confirm the importance of polymorphic codon 399 located within the BRCA1 C-terminus functional domain in XRCC1 protein (Wang et al., 2003).

The capacity to repair oxidative DNA damage was 2-fold higher among individuals with the wild-type genotype in *hOGG1* Ser326Cys as compared to those with homozygous variant genotype. The larger functional studies also suggest reduced repair function with variant alleles in *hOGG1* (Chen et al., 2003; Wang et al., 2006). Another study suggested possible explanation of decreased function of hOGG1 326Cys due to phosphorylation of the protein at 326 site that influence the sub-nuclear localisation and thereby protein function (Luna et al., 2005). It was shown that cells homozygous for the *hOGG1* Cys326 variant display reduced *in vivo* 8-oxoguanine repair rates, and the study confirmed that the lower activity of hOGG1 is associated with the oxidation of Cys326 resulting in lower enzymatic activity (Bravard et al., 2009). Our data on the *hOGG1* Ser326Cys polymorphism and the capacity to repair oxidative DNA damage may provide more quantitative data on the decrease of oxidative damage repair in association with the variant allele in the above gene.

Manuscript II

In this study, significantly higher *hOGG1* mRNA expression levels were observed among workers, suggesting an induction of *hOGG1* by styrene exposure that would be consistent with the observed lower amount of oxidation damage in white blood cells.

The *hOGG1* Ser326Cys polymorphism did not affect the levels of *hOGG1* mRNA expression and this might be considered as consistent with the functional nature of the polymorphism that affects the enzyme activity by the Ser326Cys change in the primary protein structure (Bravard et al., 2009).

To evaluate the interaction between *hOGG1* polymorphism and styrene exposure, the analysis was limited to individuals with the wild-type Ser/Ser genotype. In this subgroup of subjects, workers showed lower levels of 8-oxo-7,8-dihydroguanosine in white blood cells and significantly higher concentrations of oxidatively modified guanine derivatives in urine than controls, which may suggest that styrene exposure induce BER enzymes.

Manuscripts III and IV

The work published in manuscripts III and IV was focused on possible relevance of mRNA expression levels in DNA repair genes (*XRCC1, hOGG1, XPC*) and cell cycle genes (*TP53, p21*^{CDKN1A}*, BCL2, BAX*) in the process of genotoxicity and carcinogenesis, when studied in human population. The studied population was well characterised with respect to styrene exposure, DNA damage markers and DNA repair capacity.

The selection of DNA repair genes of interest was based on previous studies (I; Vodicka et al., 2004c), reporting the links between genotypes in *XRCC1*, *hOGG1*, and partially *XPC,* and γ-irradiation-specific DNA repair rates. The selection of cell cycle genes was based on previous *in vitro* studies assessing mRNA and protein expression levels as a response to treatment with styrene (Laffon et al., 2001; Diodovich et al., 2004).

We observed that mRNA expression levels in *XRCC1, hOGG1* and *XPC* were decreasing with increasing styrene concentration in blood and at workplace.

One of the earlier studies showed that capacity to repair irradiation specific DNA damage (reflecting mainly BER activity) was significantly higher in exposed subjects. Possible induction of repair mechanisms due to styrene exposure was proposed (Vodicka et al. 2004 a). The present results suggest that potential induction of BER most probably does not proceed via transcriptional activation. We did not discover any associations between

γ-irradiation-specific DNA repair rates and mRNA expression levels of the BER genes *XRCC1* and *hOGG1*. *XPC* mRNA expression levels decreased with increasing γ-irradiation-specific DNA repair rates on the border of significance (p=0.047). This finding is in accordance with a study that evaluated possible induction of *XPC* on transcriptional level in lymphocytes from cancer patients. High inter-individual variation in *XPC* expression was observed and *XPC* mRNA expression levels induced by ionizing radiation were substantially higher in smokers and ex-smokers (Wiebalk et al., 2007).

The expression levels of *XRCC1, hOGG1* and *XPC* were significantly lower in the exposed individuals than in the controls. There is no explanation for the exposure-related decrease of mRNA expression levels in the studied genes in humans at present. Some *in vitro* studies indicated that oxidative/genotoxic stress-generating agents do not necessarily modify the mRNA expression levels of DNA repair genes. For instance, treatment of HeLa cells with oxidative stress generating agents did not alter the mRNA expression level of *hOGG1* (Dhenaut et al., 2000). Recently, a potassium bromate-induced increase in the activity of *hOGG1* in cells was not accompanied by an increase in *hOGG1* gene expression as assessed by qPCR, suggesting a role of protein stabilization or elevated *hOGG1* catalytic activity (Mirbahai et al., 2010). A decrease of mRNA expression of *XRCC1* (to 20-40%) was recorded following the treatment with 4-nitroquinoline-1-oxide, and also UV and ionizing radiation failed to show induction of the mRNA for *XRCC1* (Yoo et al., 1992).

The increased *hOGG1* mRNA expression levels were reported with the exposure to styrene (II). The difference in mRNA expression of *hOGG1* by comparing the results in manuscript II and III may mainly be ascribed to the normalization of *hOGG1* mRNA expression to only one reference gene *GAPDH* (II). This particular reference gene was used in a previous study (Diodovich et al., 2004) and proven not to be influenced by styrene treatment. Additionally, there were differences in number of studied individuals between the two studies: the results on *hOGG1* mRNA expression in manuscripts II and III were also based on the different number of studied individuals.

The importance of normalisation of mRNA expression of a gene of interest to a reference gene was emphasized in literature. Reference gene expression has been reported to vary considerably, no systematic survey has properly determined the errors related to the common practice of using only one reference gene, nor presented an adequate way of working around this problem. Jo Vandesompele and co-workers described and validated

procedures to identify the most stable control genes in a given set of samples (Vandesompele et al., 2002). Also the use of different instruments, software, reagents, plates or seals can lead to often underestimated run-to-run differences that need to be compensated in order to make data comparable (Vermeulen et al., 2009). In 2009 the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) Guidelines were published to improve experimental practice and allow more reliable and unequivocal interpretation of qPCR results (Bustin 2009).

For an effective elimination of genotoxic burden, the cells must not necessarily increase expression of DNA repair genes at the mRNA expression level. DNA repair is a complex process, modified by DNA damage sensing and signalling (Harper and Elledge 2007), and is regulated at various levels, e.g. transcriptional regulations, post-translational modifications. Furthermore, modifications of BER proteins, including phosphorylation, acetylation, ubiquitination, etc., may also dramatically affect organelle targeting and repair activity (Fan and Wilson 2005; Hazra et al., 2007).

DNA damage markers, SSBs and SSBs endonuclease III sites increased with the increasing mRNA expression levels of *XRCC1*, *hOGG1* and *XPC*, but not with γ-irradiation-specific DNA repair rates. It is difficult to ascribe arising SSBs solely to the intermediates of DNA repair (as SSBs reflect levels of DNA strand breaks, apurinic/apyrimidinic sites as well as intermediates in the repair process (Collins 2009).

Regarding cell cycle genes, exposure related differences in mRNA gene expression levels of *TP53, p21*^{CDKN1A}*, BCL2* and *BAX* were observed in relation to occupational exposure to styrene. These results may confirm the suggested close link between DNA repair and cell cycle regulation (Branzei and Foiani 2008). The *TP53* transcriptional activities and its relationship to *p21*^{CDKN1A} have been recently reviewed, and it has been shown that *p21*^{CDKN1A} plays an essential role in DNA damage response, and regulation of this gene is complex at both mRNA and protein level (Millau et al., 2009; Cazzalini et al., 2010; Soria and Gottifredi 2010). In the exposed group we observed lower mRNA expression of *TP53*, wherein mRNA expression of *p21*^{CDKN1A} was higher in this group. A similar result was detected in PBL of workers chronically exposed to benzene, where lower mRNA expression of *TP53* was observed, but no difference was found in *p21*^{CDKN1A} mRNA expression between the exposed and control groups (Sun et al., 2009). PBL are

predominantly quiescent in G0 phase of the cell cycle. *In vitro* study on quiescent and stimulated PBL revealed that after γ-irradiation TP53 protein increased only in stimulated lymphocytes, while $p21^{CDKN1A}$ protein increased in both cases and in a dose dependent manner (Vilasova et al., 2008). Enhanced $p21^{CDKN1A}$ expression can occur through both TP53-dependent and TP53-independent mechanisms (Zhao et al., 2006; Ocker and Schneider-Stock, 2007). When studying the effect of genotoxic agents on gene expression in cell lines, the authors observed a 3-fold increase in the synthesis of the p53 protein in TK6 cells exposed to 10 μM of diepoxybutane for 24 h, but no significant elevation of *TP53* mRNA expression levels was detected under the same exposure conditions (Yadavilli et al., 2009). On the other hand, an *in vivo* assay for studying gene expression changes in epithelial cells of glandular stomach treated with N-nitroso-N-methylurea or N-methyl-N'-nitro-N-nitrosoguanidine showed up-regulation of $p21^{CDKN1A}$ mRNA expression levels in treated mice compared to controls (Okada et al., 2008). These results are in accordance with our findings of higher mRNA expression levels of $p21^{CDKN1A}$ in exposed individuals, but any direct comparison of the data from animal or experimental systems to humans should be confirmed by additional independent studies.

Both *BCL2* and *BAX* mRNA expression levels decreased with styrene exposure. This result is not in accordance with the results from the previous *in vitro* study, where over-expression of BCL2 and down-regulation of BAX was observed after styrene administration (Diodovich et al., 2004). It was also observed that the treatment of Clara cells with R-enantiomer of styrene-7,8-oxide or styrene resulted in increased *BAX/BCL2* mRNA ratio, followed by an increase in *BAX/BCL2* protein ratio, depending on the time after administration(Harvilchuck and Carlson, 2009). This discrepancy with our data may be due to the particular biological status of PBL.

Markers of DNA damage (SSBs and Endo III sites) were increasing with increasing mRNA gene expression of *BCL2* and *TP53*, whereas higher DNA damage was associated with lower mRNA expression levels of $p21^{CDKN1A}$. No association was revealed between DNA damage and mRNA expression levels of *BAX*. These correlations are difficult to explain in the light of nature of SSBs and EndoIII sites, since they do not represent exclusively markers of DNA damage, but also intermediates in the repair process (Collins 2009).

In manuscript III, it has been shown on the same population that γ-irradiation-specific DNA repair rates were the highest among individuals with low styrene exposure, followed by those with high styrene exposure and the controls. $p21^{CDKN1A}$ mRNA expression levels increased with increasing γ-irradiation-specific DNA repair capacity, particularly in the control and the low exposed groups. In the high exposed group the γ-irradiation-specific DNA repair capacity decreased in comparison to low exposed group, but the $p21^{CDKN1A}$ mRNA expression continued to rise up. This phenomenon is difficult to explain. However, $p21^{CDKN1A}$ has an active regulatory role in DNA repair. *In vitro* studies revealed the impact of $p21^{CDKN1A}$ on BER via its interactions with PCNA (Tom et al., 2001). Furthermore, $p21^{CDKN1A}$ was shown to bind with PARP-1 and regulate the interaction between PARP-1 and BER factors, wherein $p21^{CDKN1A}$ is required for the turnover of PARP-1 association with XRCC1 and DNA polymerase β, to promote efficient repair (Frouin et al., 2003; Cazzalini et al., 2010). We may suggest that our finding about the relationship between DNA repair capacity and $p21^{CDKN1A}$ mRNA expression level is in line with above-mentioned studies and confirms the pivotal role of $p21^{CDKN1A}$ in the DNA repair.

6. CONCLUSIONS

1. Evaluation of the association between SNPs in selected DNA repair genes and their mRNA expression levels in control individuals and individuals occupationally exposed to styrene.

In manuscript II, no association was detected between *hOGG1* mRNA expression and *hOGG1* Ser326Cys polymorphism. On the study population from manuscripts III and IV, possible associations between SPNs in *XRCC1* Arg399Gln, *XRCC1* Arg280His *XRCC1*Arg194Trp, *hOGG1* Ser326Cys and *XPC* Lys939Gln, and mRNA expression levels of *XRCC1, hOGG1* and *XPC* were analysed, however no association was revealed (data not shown). In study on healthy subjects, it was shown that SNPs in *XPD* significantly influenced its mRNA expression in lymphocytes and also had an effect on mRNA secondary structure (Wolfe et al., 2007). Investigation of an effect of SNPs on mRNA expression may be substantially influenced by a size of study population, by the overall mode of analysis.

2. *Evaluation of mRNA expression levels in selected DNA repair genes and genes of cell cycle control in styrene exposed and control individuals with respect to markers of occupational exposure to styrene*.

In manuscript II, significantly higher *hOGG1* mRNA expression levels were observed among workers, suggesting an induction of *hOGG1* by styrene exposure.

In manuscripts III and IV we reported that mRNA expression levels of *XRCC1, XPC* and *hOGG1* decreased with increasing concentration of styrene in blood and at workplace. However, there is no simple mechanistic explanation for an exposure related decrease in mRNA expression levels of *XRCC1, XPC* and *hOGG1* in humans at present. *BAX, BCL2* and *TP53* mRNA expression levels were decreasing with increasing concentration of styrene at workplace, styrene in blood, styrene in exhaled air. $p21^{CDKN1A}$ mRNA expression level positively correlated with styrene at workplace, styrene in blood, styrene in exhaled air, occupation in years.

3. *Evaluation of mRNA expression levels in selected DNA repair genes and genes of cell cycle control in styrene exposed and control individuals with respect to DNA damage and phenotypic outcome of BER.*

In manuscript III, we observed that both markers of DNA damage (SSBs and SSBs endonuclease III sites) increased with the increasing mRNA expression levels of *XRCC1*, *hOGG1* and *XPC*; and expression levels of *XPC* negatively correlated with γ-irradiation-specific DNA repair capacity.

Interestingly, $p21^{CDKN1A}$ mRNA expression negatively correlates with SSBs and EndoIII sites and positively correlates with irradiation specific DNA repair rates, suggesting important regulation role of this gene in BER. On the other hand, *TP53* mRNA expression positively correlates with SSBs and EndoIII sites, but does not correlate with irradiation specific DNA repair rates. The mRNA expression profiles of *BAX* and *BCL2* do not show any clear and unambiguous link to the other studied markers (Manuscript IV).

Most interestingly, we did not find any association between irradiation specific DNA repair rates and mRNA expression levels of *XRCC1* and *hOGG1*. DNA repair process may be regulated and modified at various levels, for instance by post-translational modifications that may enhance protein activity and stability.

4. Overall evaluation of acquired data with respect to genotoxicity and molecular mechanisms of cell response to styrene exposure and DNA damage

Based on manuscripts I - IV we may conclude that although DNA repair polymorphisms may affect the overall DNA repair capacity and/or the cancer risk, simple mechanistic links may not be applicable. For an effective elimination of genotoxic burden, the cells may not necessarily increase expression of DNA repair genes at transcriptional level. The unambiguous evidence for the DNA repair induction as a response to styrene-induced genotoxicity was not obtained. Negative correlations of mRNA expression levels of studied genes (*XRCC1, hOGG1* and *XPC*) and DNA damage with styrene exposure would require further, highly-targeted studies, supported by analyses on protein levels. It also concerns *TP53* and $p21^{CDKN1A}$ regulation as a part of DDR response, which is highly complex and cell type- and cell state-dependent.

Based on the above discussed results, occupational exposure to styrene exerts an impact on gene expression at mRNA levels of the studied genes in lymphocytes. So far, the existing literature does not provide any clear evidence about a possible links between mRNA expression levels of the studied genes, response to DNA damage and response to occupational exposure in humans. The relationship between styrene exposure, DNA

repair and mRNA expression levels of DNA repair and cell cycle control genes presented an interesting *in vivo* model to investigate the basic principles of cellular regulation. We consider these points addressing the capacity of individual to maintain genomic stability as critical issues in carcinogenesis.

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8. MANUSCRIPTS I-IV IN EXTENSO

Manuscript I

Vodicka P, Stetina R, Polakova V, Tulupova E, Naccarati A, Vodickova L, Kumar R, **Hanova M**, Pardini B, Slyskova J, Musak L, De Palma G, Soucek P, Hemminki K

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Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects

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We investigated association between polymorphisms in DNA repair genes and the capacity to repair DNA damage induced by γ-irradiation and by base oxidation in a healthy population. Irradiation-specific DNA repair rates were significantly decreased in individuals with *XRCC1* Arg399Gln homozygous variant genotype (0.45 ± 0.47 SSB/10^9 Da) than in those with wild-type genotype (1.10 ± 0.70 SSB/10^9 Da, $P = 0.0006$, Mann–Witney *U*-test). The capacity to repair oxidative DNA damage was significantly decreased among individuals with *hOGG1* Ser326Cys homozygous variant genotype (0.37 ± 0.28 SSB/10^9 Da) compared to those with wild-type genotype (0.83 ± 0.79 SSB/10^9 Da, $P = 0.008$, Mann–Witney *U*-test). Investigation of genotype combinations showed that the increasing number of variant alleles for both *XRCC1* Arg399Gln and *APE1* Asn148Glu polymorphisms resulted in a significant decrease of irradiation-specific repair rates ($P = 0.008$, Kruskal–Wallis test). Irradiation-specific DNA repair rates also decreased with increasing number of variant alleles in *XRCC1* Arg399Gln in combination with variant alleles for two other *XRCC1* polymorphisms, Arg194Trp and Arg280His ($P = 0.002$ and $P = 0.005$, respectively; Kruskal–Wallis test). In a binary combination variant alleles of *hOGG1* Ser326Cys and *APE1* Asn148Glu polymorphisms were associated with a significant decrease in the capacity to repair DNA oxidative damage ($P = 0.018$, Kruskal–Wallis test). In summary, *XRCC1* Arg399Gln and *hOGG1* Ser326Cys polymorphisms seem to exert the predominant modulating effect on irradiation-specific DNA repair capacity and the capacity to repair DNA oxidative damage, respectively.

Abbreviations: BER, base excision DNA repair; SSB, single-strand breaks; SNP, single nucleotide polymorphism; PBL, peripheral blood lymphocytes.

Introduction

In the recent years, several studies have investigated polymorphisms in DNA repair genes and their possible links to the risk of various cancers. Sequence variants in DNA repair genes are assumed to modulate DNA repair capacity and, therefore, are associated with the altered cancer risk. As an example, the *hOGG1* Cys/Cys genotype has been associated with an increased lung cancer risk (1). In tobacco-related cancers a protective effect of *XRCC1* Arg194Trp variant allele was shown, while variant allele in *XRCC1* Arg399Gln polymorphism was associated with an increased risk among light smokers only (2). An increased risk of colorectal cancer was recently reported for *XRCC1* Arg399Gln variant allele (3). Statistically significant associations have been found between *XPD* polymorphisms and skin, breast and lung cancers [reviewed by ref. (4)]. Increasing number of studies relating genetic polymorphisms in DNA repair genes and various kinds of cancer in the past 5 years do not provide unambiguous consistent associations, mainly due to low statistical power for detecting a moderate effect, false-positive results, heterogeneity across study populations (5), failure to consider effect modifiers such as environmental exposures (6) and, most importantly, due to the virtually unknown relationship between the genotype and the functional outcome (phenotype) (7).

An analysis of SNPs in 88 DNA repair genes and their functional evaluation, based on the conservation of amino acids among the protein family members, shows that ~30% of variants of DNA repair proteins are likely to affect substantially the protein function. It applies particularly for polymorphisms in *XRCC1* Arg280His and Arg399Gln, and *XRCC3* Thr241Met (8). Susceptibility towards ionizing radiation, as measured by prolonged cell cycle G_2 delay, was determined in relation to *XRCC1* Arg194Trp, Arg399Gln and *APE1* Asn148Glu genotypes. Ionizing radiation sensitivity was significantly affected by amino acid substitution variants in both *XRCC1* and *APE1* genes (9). Using the cytogenetic challenge assay, *XRCC1* 399Gln and *XRCC3* 241Met alleles were associated with significant increase in chromosomal deletions as compared with the corresponding homozygous wild-types. Authors concluded that *XRCC1* 399Gln and *XRCC3* 241Met are significantly defective in base excision repair (BER), while *XPD* 312Asn and *XPD* 751Gln are significantly defective in nucleotide excision repair (NER) (10). Individuals with the wild-type Arg/Arg genotype in *XRCC1* Arg194Trp polymorphism exhibited significantly higher values of chromosomal breaks, as assessed by the mutagen sensitivity assay, than those with variant Trp allele, suggesting a protective effect of this allele. On the other hand, variant Gln allele in *XRCC1* Arg399Gln was significantly associated with an increase in chromosomal breaks per cell. These data are biologically plausible, since codon 399 is located within the *BRCA1* C-terminus

functional domain and codon 194 is in the linker region of the *XRCC1* N-terminal functional domain (11). Three studies using different approaches have found a functional impact of *hOGG1* Ser326Cys polymorphism (12–14), but other studies [reviewed by ref. (1)] did not find any conclusive result for *hOGG1* genetic polymorphisms. *hOGG1* Ser326Cys polymorphism has also been described to affect the glycosylase function due to the localization and phosphorylation status (15). The results of such tests allow a more meaningful choice of genes for association studies, though they are still not sufficient for an accurate prediction for the DNA repair capacity.

In the present report we attempt to investigate associations between DNA repair genetic polymorphisms (*XPD* Lys751Gln, *XPG* Asn1104His, *XPC* Lys939Gln, *XRCC1* Arg194Trp, Arg280His and Arg399Gln, *APE1* Asn148Glu, *hOGG1* Ser326Cys *XRCC3* Thr241Met and *NBS1* Glu185Gln) and individual DNA repair activity in a general healthy population from the Central Europe, assessing *in vitro* the capacity to repair both irradiation-specific induced- and oxidative-induced DNA damage. In the former case, the comet assay (single cell alkaline gel electrophoresis) has been modified to measure the ability of lymphocytes to repair γ-irradiation induced single-strand breaks (SSBs) after 40 min of incubation (16), and in the latter, to measure the ability of a subcellular extract of lymphocytes to carry out the initial incision step of repair on a DNA substrate carrying specific lesions-namely, oxidized bases introduced by visible light in the presence of photosensitizer (17).

Materials and methods

Study population

The study was conducted on 244 healthy individuals (183 men and 61 women, mean age 41.3 ± 11.3 years, 90 individuals were smokers and 154 non-smokers) employed in local administration, medical centers and various branches of plastic industry. The investigated population was recruited in the regions of western Slovakia and eastern Bohemia, which exhibit close similarities in socio-economical conditions. Confounding factors, like X-rays, medical drug treatments, dietary (vitamins intake, particular diets) and lifestyle habits (smoking, alcohol and coffee consumptions) and possible exposure-related effects were recorded in detailed questionnaires and considered in the statistical analyses. Present cohort is representative, ethnically homogenous population and therefore suitable for the determination of relationships between DNA repair genetic polymorphisms and DNA repair rates. Lower number of observations for DNA repair rates, in comparison to that given in Table I, were due to methodological limitations (i.e. successful processing of the fresh material). The study design was approved by the local Ethical Committee and the participants provided their informed consent to be included in the study. The sampling of blood was carried out according to the Helsinki Declaration.

DNA repair polymorphisms

Single nucleotide polymorphisms (SNPs) in genes encoding DNA repair enzymes were determined by a PCR–RFLP based method. PCR products were generated using 50 ng of genomic DNA in 25 µl volume reactions containing 20 mM Tris–HCl, 50 mM KCl, 2.0 mM $MgCl_2$, 0.3 mM each dNTP, 0.3 µM each primer (Table I) and 0.2 U *Taq* DNA polymerase. The temperature conditions for PCR were established as denaturation at 94°C for 30 s, annealing (given in Table I) for 30 s, elongation at 72°C for 30 s and final extension at 72°C for 5 min. The amplified fragments were digested with appropriate restriction endonucleases (Table I) and analyzed. The digested PCR products were resolved on 3% agarose gels containing ethidium bromide and visualized under UV light. The genotype results were regularly confirmed by re-genotyping (10% of samples) and by TaqMan allelic discrimination assay (Assay-on-Demand', Applied Biosystems, Foster City, USA), using Real-Time Gene Amp PCR system on AB 7500 equipment (Applied Biosystems, Foster City, USA). The concordance rate was 100%.

γ-irradiation DNA repair test

Peripheral blood lymphocytes (PBL), isolated using Ficol gradient, were used to test individual DNA repair capacity as described previously (18,19). Briefly, cells embedded in agarose on slides were irradiated with 5 Gy of γ-rays (0.42 Gy/min) and either lysed immediately or incubated at 37°C for 40 min before the lysis. The DNA breaks induced by γ-rays are repaired during the 40 min of incubation period, according to the individual repair capacity. The results (i.e. the amount of repaired SSBs) are calculated as a difference between the initial levels of SSBs, measured immediately after irradiation, and the levels of SSBs detected after 40 min of incubation. The repaired DNA damage is subsequently expressed as SSB/10^9 Da. Consequently, higher values of repaired SSBs reflect higher DNA repair activity. The detailed description of the tentative origin γ-ray-induced DNA

Table I. Details on investigated SNPs in DNA repair genes

Genetic polymorphism	Exon	Primer sequence	Annealing temp. (°C)	Restriction enzyme	Genotype distribution	Frequency of variant allele
Base-excision repair						
XRCC1 Arg194Trp	6	F GCC CCG TCC CAG GTA R AGC CCC AAG ACC CTT TCA CT	63	*Msp*I	CC TC TT 184 30 2	qT = 0.078
XRCC1 Arg280His	9	F TTG ACC CCC AGT GGT GCT R CCC TGA AGG ATC TTC CCC AGC	57	*Rsa*I	GG GA AA 202 13 1	qA = 0.035
XRCC1 Arg399Gln	10	F GCC CCT CAG ATC ACA CCT AAC R CAT TGC CCA GCA CAG GAT AA	65	*Msp*I	GG GA AA 104 112 18	qA = 0.316
hOGG1 Ser326Cys	7	F AGT GGA TTC TCA TTG CCT TCG R GGT GCT TGG GGA ATT TCT TT	59	*Fnu*4HI	CC CG GG 154 75 13	qG = 0.209
APE1 Asn148Glu	5	F CTG TTT CAT TTC TAT AGG CTA R AGG AAC TTG CGA AAG GCT TC	59	*Bfa*I	TT TG GG 88 112 35	qG = 0.387
Nucleotide-excision repair						
XPD Lys751Gln	23	F CCC CTC TCC CTT TCC TCT GTT R GCT GCC TTC TCC TGC GAT TA	60	*Pst*I	AA AC CC 65 138 36	qC = 0.439
XPG Asn1104His	15	F TGG ATT TTT GGG GGA GAC CT R CGG GAG CTT CCT TCA CTG AGT	56	*Hsp*92II	GG GC CC 114 102 11	qC = 0.273
XPC Lys939Gln	15	F GAT GCA GGA GGT GGA CTC TCT R GTA GTG GGG CAG CAG CAA CT	61	*Pvu*II	AA AC CC 83 110 41	qC = 0.410
Double-strand break repair						
XRCC3 Thr241Met	7	F GCT CGC CTG GTG GTC ATC R CTT CCG CAT CCT GGC TAA AAA	59	*Hsp*92II	CC CT TT 71 121 36	qT = 0.423
NBS1 Glu185Gln	5	F GGA TGT AAA CAG CCT CTT G R CAC AGC AAC TAT TAC ATC CT	59	*Hinf*I	GG GC CC 89 122 25	qC = 0.364

damage as well as the calibration and optimization of the repair test have already been described in details elsewhere (17).

Oxidative DNA repair test

The repair capacity of PBL extracts towards repairing 8-oxoguanine was determined as previously described (20). Briefly, isolated lymphocytes from each individual were collected and divided into aliquots and stored in liquid nitrogen at −80°C, until experiment. Before an assay, a frozen aliquot was thawed and washed with 1% Triton X-100 in a lysis buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, pH 7.8) and the lysate was centrifuged to remove nuclei and cell debris. The supernatant was mixed with a reaction buffer (45 mM HEPES, 0.25 mM EDTA, 2% glycerol, 0.3 mg/ml BSA pH 7.8) and kept on ice until use.

A substrate of HeLa cells (2×10^5 per dish) was prepared and pretreated with 2 ml 0.1 μM phosphosensitizer Ro 19-8022 (Hoffmann-La Roche, Basel, Switzerland) and PBSG, and irradiated with a fluorescent lamp (2 min on ice from a 1000 W tungsten halogen lamp, to induce 8-oxoguanines). HeLa cells were successively washed, removed from dishes by gentle trypsinization and embedded in agarose on slides and placed in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris made to pH 10 with NaOH, and 1% Triton X-100) for 1 h at 4°C. After lysis slides are incubated either with individual PBL extracts or with buffer alone at 37°C for 45 min, followed by electrophoresis and neutralization according to comet assay standard protocol (20).

The results (i.e. the amount of repaired oxidative DNA damage, reflecting the removal of 8-oxoguanines) are calculated as a difference between the levels of SSBs, measured in slides with PBL extract and the levels of SSBs measured in slides with buffer only. The level of SSBs, is expressed as SSB/10^9 Da.

Statistical analyses

Statistical calculations were performed using Statgraphics, version 7 (Manugistics Inc., Cambridge, MA). Hardy–Weinberg equilibrium was tested using the chi-square 'goodness-of-fit' test. The data for both DNA repair assays, given in Tables II–VI, are expressed as mean ± SD. For testing significant differences between groups, specifically Table II, the non-parametrical Mann–Whitney *U*-test was applied. Associations between the combined genotypes and DNA repair rates were tested by Kruskal–Wallis test (as shown in Tables III–VI). Simple linear regression analysis was used to estimate the correlation between confounder and DNA repair rates, whereas multifactorial regression analysis was applied to discern the major influencing factors on the DNA repair rates (i.e. analyzing main confounding factors and DNA repair polymorphisms simultaneously).

For statistical analyses non-smokers as well as males were assigned as '0', while smokers and females as '1', age was calculated as continuous variable. Similarly, for statistical analyses the wild-type genotype was assigned as '0', heterozygous variant allele bearers as '1' and homozygous variant allele bearers as '2'.

Evaluation of DNA repair rates in relation to gene–gene interactions, when three and more polymorphisms were considered, was based on the construction of arbitrary score for variant allele. Wild-type allele was assigned as '0', heterozygous variant allele as '1' and homozygous variant allele as '2'. The above approach does not discriminate all possible genotype combinations (i.e. 27 theoretically possible outcomes in ternary and 81 in quaternary combinations), but takes into account a number of variant alleles in particular combination and the higher score reflects the higher number of variant alleles in either genes.

Results

Genotype distribution

The genotype distributions for individual DNA repair genes are shown in Table I. Allelic frequencies in *XPD* Lys751Gln, *XPG* Asn1104His, *XPC* Lys939Gln, *XRCC1* Arg399Gln, *XRCC3* Thr241Met, *NBS1* Glu185Gln and *APE1* Asn148Glu are in agreement with those earlier described for the central European population (21,22), while allelic frequencies in *hOGG1* Ser326Cys, *XRCC1* Arg194Trp and Arg280His for the same population are shown for the first time (Table I). The genotype distribution for all investigated polymorphisms, except for *XPD* Lys751Gln ($\chi^2 = 7.0$, $P = 0.01$), was in the Hardy–Weinberg equilibrium.

Table II. Irradiation-specific DNA repair rates and oxidative DNA damage repair rates stratified for individual DNA repair polymorphisms

Genotypes	Irradiation-specific DNA repair rates (SSNs/10^9 Da)	Oxidative DNA damage repair rates (SSBs/10^9 Da)
XRCC1 Arg399Gln		
GG	1.10 ± 0.70[a] ($n = 92$)	0.75 ± 0.69 ($n = 88$)
GA	0.76 ± 0.69[a] ($n = 103$)	0.76 ± 0.87 ($n = 95$)
AA	0.45 ± 0.47 ($n = 17$)	0.75 ± 0.41 ($n = 14$)
XRCC1 Arg280His		
GG	0.86 ± 0.74 ($n = 183$)	0.72 ± 0.69 ($n = 169$)
GA	0.90 ± 0.65 ($n = 12$)	0.44 ± 0.49 ($n = 12$)
AA	1.34 ($n = 1$)	0.5 ($n = 1$)
XRCC1 Arg194Trp		
CC	0.88 ± 0.73 ($n = 167$)	0.68 ± 0.63 ($n = 155$)
CT	0.87 ± 0.74 ($n = 27$)	0.77 ± 0.95 ($n = 26$)
TT	0.89 ± 0.72 ($n = 2$)	0 ($n = 1$)
APE1 Asn148Glu		
TT	0.95 ± 0.82 ($n = 75$)	0.79 ± 0.85 ($n = 72$)
TG	0.83 ± 0.65 ($n = 100$)	0.73 ± 0.68 ($n = 96$)
GG	0.86 ± 0.61 ($n = 32$)	0.68 ± 0.69 ($n = 26$)
hOGG1 Ser326Cys		
CC	0.88 ± 0.68 ($n = 143$)	0.83 ± 0.79[a] ($n = 130$)
CG	0.90 ± 0.78 ($n = 63$)	0.61 ± 0.67[a] ($n = 64$)
GG	0.66 ± 0.76 ($n = 12$)	0.37 ± 0.28 ($n = 13$)
XPD Lys751Gln		
AA	0.87 ± 0.63 ($n = 57$)	0.61 ± 0.66 ($n = 55$)
AC	0.86 ± 0.72 ($n = 123$)	0.86 ± 0.84 ($n = 119$)
CC	0.91 ± 0.81 ($n = 35$)	0.52 ± 0.47 ($n = 29$)
XPG Asn1104His		
GG	0.83 ± 0.70 ($n = 98$)	0.79 ± 0.80 ($n = 90$)
GC	0.94 ± 0.73 ($n = 95$)	0.72 ± 0.72 ($n = 93$)
CC	0.83 ± 0.45 ($n = 11$)	0.76 ± 1.01 ($n = 10$)
XPC Lys939Gln		
AA	0.82 ± 0.70 ($n = 77$)	0.86 ± 0.87 ($n = 67$)
AC	0.86 ± 0.65 ($n = 96$)	0.68 ± 0.69 ($n = 94$)
CC	1.00 ± 0.86 ($n = 36$)	0.73 ± 0.72 ($n = 38$)
XRCC3 Thr241Met		
CC	0.89 ± 0.66 ($n = 65$)	0.79 ± 0.69 ($n = 57$)
CT	0.85 ± 0.70 ($n = 105$)	0.76 ± 0.79 ($n = 101$)
TT	0.77 ± 0.74 ($n = 34$)	0.73 ± 0.84 ($n = 35$)
NBS1 Glu185Gln		
GG	0.89 ± 0.60 ($n = 79$)	0.82 ± 0.75 ($n = 73$)
GC	0.81 ± 0.72 ($n = 111$)	0.66 ± 0.69 ($n = 102$)
CC	1.12 ± 0.99 ($n = 23$)	0.68 ± 0.72 ($n = 25$)

The results are presented as mean ± SD.
[a]$P < 0.05$. The comparison between individual genotypes was performed by Mann–Whitney *U*-test.

DNA repair rates and confounders

Both irradiation-specific DNA repair rates and the capacity to repair of oxidative DNA damage were not affected by age and there was no significant difference in both DNA repair rates between men and women. Irradiation-specific DNA repair rates were significantly higher among smokers (1.05 ± 0.81 SSB/10^9 Da) as compared to non-smokers (0.77 ± 0.62 SSB/10^9 Da, $P = 0.014$, Mann–Whitney *U*-test), while the capacity to repair of oxidative DNA damage was not affected by smoking habit. By investigating simultaneous influence of genotypes in DNA repair and recorded confounders (age, sex, exposure status and smoking), irradiation-specific DNA repair rates were mainly affected by polymorphism in *XRCC1* Arg399Gln gene ($t = -4.54$, $P < 0.001$), and also by smoking ($t = 2.92$, $P = 0.004$, $R^2 = 0.132$; multiple regression analysis). Figure 1 shows the lowest irradiation-specific DNA repair rates being associated with homozygous variant *AA XRCC1* Arg399Gln genotype both in smokers and non-smokers, although only in non-smokers the difference in

Table III. Effect of selected binary combinations of SNPs of BER genes on irradiation-specific DNA repair rates (expressed as SSBs/10^9 Da)

	APE1 Asn148Glu genotype			
	TT	TG	GG	χ^2, *P*
(A) *XRCC1* Arg399Gln genotype				
GG	1.14 ± 0.77 (46)	1.00 ± 0.67 (30)	1.10 ± 0.59 (14)	0.77, 0.682
GA	0.81 ± 0.86 (28)	0.78 ± 0.64 (58)	0.68 ± .059 (17)	0.37, 0.831
AA	0.57 ± 0.43 (6)	0.42 ± 0.47 (12)	0.28 ± 0.28 (2)	0.96, 0.619
χ^2, *P*	6.98, 0.03	8.35, 0.015	3.76, 0.154	Overall $\chi^2 = 20.87$, $P = 0.0008$
	XRCC1 Arg280His genotype			
	GG	GA	AA	χ^2, *P*
(B) *XRCC1* Arg399Gln genotype				
GG	1.11 ± 0.73 (74)	0.98 ± 0.80 (7)	1.34 (1)	0.43, 0.807
GA	0.75 ± 0.72 (91)	0.84 ± 0.47 (4)	— (0)	0.20, 0.652
AA	0.44 ± 0.48 (16)	0.60 ± 0.06 (2)	— (0)	0.34, 0.563
χ^2, *P*	18.32, 0.0001	0.94, 0.627	—	Overall $\chi^2 = 14.91$, $P = 0.002$
	XRCC1 Arg194Trp genotype			
	CC	CT	TT	χ^2, *P*
(C) *XRCC1* Arg399Gln genotype				
GG	1.12 ± 0.73 (71)	1.00 ± 0.83 (10)	1.40 (1)	0.68, 0.711
GA	0.74 ± 0.73 (91)	0.81 ± 0.64 (18)	— (0)	0.20, 0.651
AA	0.49 ± 0.47 (16)	0.00 (1)	0.38 (1)	1.22, 0.543
χ^2, *P*	16.90, 0.0001	2.27, 0.321	1.00, 0.317	Overall $\chi^2 = 15.00$, $P = 0.005$

The results are presented as mean ± SD; in parentheses are reported the number of individuals with the particular genotype combination. Comparisons were performed by Kruskal–Wallis test.

comparison to wild-type *GG* genotype was statistically significant.

DNA repair rates and genotype analyses

Irradiation-specific DNA repair rates were significantly decreased in individuals with the homozygous variant (*AA*) in *XRCC1* Arg399Gln than those with the wild-type (*GG*) and heterozygous (*GA*) genotypes, (Mann–Whitney *U*-test: $P = 0.0006$ and $P = 0.002$, respectively; Table II).

We did not observe any significant influence on irradiation-specific DNA repair rates in the *XRCC1* Arg194Trp and Arg280His and *APE1* Asn148Glu polymorphisms. Similarly, no association between the genetic polymorphism in *hOGG1* Ser326Cys and irradiation-specific DNA repair rates was observed (Table II).

Our results did not show any effect on irradiation-specific DNA repair rates by genetic polymorphisms in genes involved either in NER (*XPD* Lys751Gln, *XPG* Asn1104His and *XPC* Lys939Gln) or DNA recombination repair (*XRCC3* Thr241Met and *NBS1* Glu185Gln) (Table II).

Combinations of different polymorphisms in BER genes were investigated in relation to irradiation-specific DNA repair rates. By testing all genotype combinations of *XRCC1* Arg399Gln and *APE1* Asn148Glu the irradiation-specific repair rates significantly decreased with increasing number of variant (*A*) allele in *XRCC1* Arg399Gln, whereas *APE1* Asn148Glu genotype contributed moderately (Kruskal–Wallis test: $\chi^2 = 20.87$, $P = 0.008$, Table III).

A significant decrease in irradiation-specific DNA repair rates was also constantly observed in association with variant allele (*A*) in *XRCC1* Arg399Gln, whereas no contribution of the two other investigated polymorphisms in *XRCC1* gene (Arg280His and Arg194Trp) was observed [Kruskal–Wallis test: $\chi^2 = 14.91$, $P = 0.002$ and $\chi^2 = 15.00$, $P = 0.005$, respectively; (Table III, B and C)].

When *XRCC1* Arg399Gln polymorphism was not taken into consideration, binary genotype combinations in BER genes did not significantly affect the level of irradiation-specific DNA repair rates (data not shown).

Similar results were observed for combinations of 3 and 4 polymorphisms in BER genes assessed using a score system that reflects the number of variant alleles in particular combination (the data are shown in Table V), revealing again that the predominant effect on the irradiation-specific DNA repair rates is associated with variant (*A*) allele in *XRCC1* Arg399Gln, and this significant tendency persists in spite of increasing number of genes analyzed in combination.

The capacities to repair oxidative DNA damage were significantly decreased in individuals with the homozygous variant (*GG*) genotype in *hOGG1* Ser326Cys as compared to those with wild-type (*CC*) and heterozygous (*CG*) genotypes (Mann–Whitney *U*-test: $P = 0.008$ and $P = 0.041$, respectively; Table II).

A significant decrease in the capacity to repair DNA oxidative damage was also associated with combination of variant alleles in *hOGG1* Ser326Cys and *APE1* Asn148Glu, (Kruskal–Wallis test: $\chi^2 = 8.84$, $P = 0.018$, Table IV). As evident from Table IV, the predominant effect is due to the variant *G* allele in *hOGG1* Ser326Cys.

Table IV. Effect of a selected binary combination of SNPs of BER genes on the capacity to repair oxidative DNA damage (expressed as SSBs/10^9 Da)

	APE1 Asn148Glu genotype			
	TT	TG	GG	χ^2, *P*
(A) *hOGG1* Ser326Cys genotype				
CC	0.84 ± 0.83 (48)	0.81 ± 0.75 (59)	0.63 ± 0.46 (19)	0.62, 0.734
CG	0.60 ± 0.78 (22)	0.61 ± 0.54 (30)	0.51 ± 0.39 (8)	0.85, 0.654
GG	0.54 ± 0.20 (5)	0.48 ± 0.36 (6)	0.23 ± 0.23 (2)	1.25, 0.535
χ^2, P	4.43, 0.097	4.10, 0.129	1.80, 0.408	Overall $\chi^2 = 8.84$, $P = 0.018$
	XRCC1 Arg194Trp genotype			
	CC	CT	TT	χ^2, *P*
(B) *hOGG1* Ser326Cys genotype				
CC	0.71 ± 0.59 (95)	0.96 ± 1.00 (20)	0.00(1)	2.96, 0.227
CG	0.66 ± 0.31 (50)	0.35 ± 0.48 (6)	— (0)	1.21, 0.271
GG	0.38 ± 0.31 (10)	0.28 ± 0.21 (2)	— (0)	0.06, 0.830
χ^2, *P*	4.02, 0.094	3.37, 0.186	—	Overall $\chi^2 = 8.85$, $P = 0.045$
	XRCC1 Arg280His genotype			
	GG	GA	AA	χ^2, *P*
(C) *hOGG1* Ser326Cys genotype				
CC	0.78 ± 0.69 (108)	0.26 ± 0.23 (7)	0.50 (1)	6.09, 0.048
CG	0.63 ± 0.71 (51)	0.68 ± 0.69 (5)	— (0)	0.01, 0.920
GG	0.36 ± 0.29 (12)	— (0)	— (0)	—
χ^2, *P*	7.71, 0.021	0.83, 0.370	—	Overall $\chi^2 = 8.13$, $P = 0.017$

The results are presented as mean ± SD; in parentheses are reported the number of individuals with the particular genotype combination. Comparisons were performed by Kruskal–Wallis test.

Binary combinations of polymorphisms in *hOGG1* Ser326Cys and *XRCC1* Arg194Trp, and Arg280His showed that the predominance of the variant *G* allele in *hOGG1* Ser326Cys is associated with the lower capacity to repair DNA oxidative damage (Tables IV and IV).

By testing the effect of all analyzed polymorphisms in BER genes, the capacity to repair DNA oxidative damage decreased with increasing number of variant alleles in *hOGG1* Ser326Cys in combination with the increasing number of variant alleles in the other investigated polymorphisms (Kruskal–Wallis test: $\chi^2 = 11.07$, $P = 0.050$, Table VI).

Discussion

Age-related decrease has been observed for hOGG1 activity in PBL from healthy individuals (13) as well as for irradiation-specific repair rates (16), whereas both irradiation-specific DNA repair rates and the capacity for the repair of oxidative DNA damage were not affected by age in our study. Interestingly, irradiation-specific DNA but not oxidative DNA damage-related DNA repair rates were significantly higher in smokers than non-smokers. In previous studies xenobiotic exposure-related increase in BER capacities has been recorded in individuals occupationally exposed to styrene (16) and xenobiotics in the tire plant, suggesting possible induction of DNA repair (23). Approximately 2-fold higher irradiation-specific DNA repair rates were found in smokers than in non-smokers (23). These findings may have be consequential, since exposure to potentially carcinogenic industrial chemicals as well as to the complex mixture of carcinogens in cigarette smoke seems to result in an increased BER capacity in healthy, cancer-free population. Whether this increase is due to an induction, or to a process of adaptation, remains to be clarified.

Irradiation-specific DNA repair rates were significantly higher among individuals with the wild-type genotype in *XRCC1* Arg399Gln as compared to those with homozygous variant genotype. Because most of the DNA damage induced by γ-irradiation is repaired in a short time (<1 h), the measured DNA repair activity is attributable mainly to the BER pathway (16,18), in agreement with the role of the *XRCC1* gene. An observation of the decreased DNA repair capacity in individuals bearing variant *A* allele in *XRCC1* exon 10 (codon 399) is additionally supported by the cytogenetic challenge assay (10), protein conservation analysis (8) and by increased irradiation sensitivity (9). These data seem to be biologically plausible, as XRCC1 protein acts as a coordinator of single strand break repair proteins in the base excision repair pathway with polymorphic codon 399 located within the *BRCA1* C-terminus functional domain (11). By testing the effect of other genetic polymorphisms in individual genes involved in BER, i.e. *XRCC1* Arg194Trp and Arg280His and *APE1* Asn148Glu, we did not observe any significant influence on irradiation-specific DNA repair rates. Although the highest DNA repair rate was seen in just one individual with homozygous variant genotype in *XRCC1* Arg280His, no conclusion may be drawn on the base of our present study. On the contrary, irradiation hypersensitivity was observed in 135 women with homozygous variant Glu/

Table V. Irradiation-specific DNA repair rates in relation to combinations of SNPs in BER genes[a]

Score	*n*	Irradiation-specific DNA repair rates (SSBs/10^9Da)
(A) *XRCC1* Arg399Gln, Arg280His and Arg194Trp		
0	66	1.14 ± 0.71
1	90	0.77 ± 0.75
2	36	0.72 ± 0.60
3	3	0.40 ± 0.35
4	1	0.38
$\chi^2 = 15.29$, $P = 0.004$		
(B) *XRCC1* Arg399Gln, Arg280His and *APE1* Asn148Glu		
0	37	1.20 ± 0.81
1	51	0.83 ± 0.79
2	75	0.87 ± 0.65
3	33	0.55 ± 0.52
4	2	0.18 ± 0.18
5	1	0.38
$\chi^2 = 16.81$, $P = 0.005$		
(C) *XRCC1* Arg399Gln, Arg194Trp and *APE1* Asn148Glu		
0	33	1.22 ± 0.82
1	55	0.92 ± 0.82
2	71	0.82 ± 0.60
3	28	0.55 ± 0.60
4	7	0.55 ± 0.45
5	1	0.38
$\chi^2 = 14.94$, $P = 0.011$		
(D) *XRCC1* Arg399Gln, Arg280His, Arg194Trp and *APE1* Asn148Glu		
0	29	1.23 ± 0.83
1	52	0.88 ± 0.84
2	71	0.87 ± 0.62
3	34	0.60 ± 0.57
4	7	0.54 ± 0.50
5	2	0.47 ± 0.13
$\chi^2 = 12.77$, $P = 0.026$		

The results are presented as mean ± SD. Comparisons were performed by Kruskal-Wallis test.
[a]Combinations were constructed on the base of an arbitrary score for variant alleles (See Materials and Methods for details). The higher score means the higher number of variant alleles in combined SNPs.

Glu genotype in *APE1* (9). It becomes more apparent in the light of the occurrence of the variant allele in the general population, which slightly exceeds 3%. The functional significance of *XRCC1* Arg280His polymorphism is not yet known (2). The data from the literature indicate that individuals with the wild-type Arg/Arg genotype in *XRCC1* Arg280His exhibit significantly higher chromosomal breaks per cell than those with variant His allele. We did not observe any association of polymorphism in codon 194 in *XRCC1* Arg194Trp, probably due to low occurrence of the variant allele in our studied population (29 individuals with at least one variant allele). The lack of observed effect of *APE1* polymorphism on BER is in agreement with the outcome of computational functional test, which suggested that this SNP is unlikely to exhibit an effect on the protein function (8).

Table VI. The capacity to repair oxidative DNA damage in relation to combinations of SNPs in BER genes[a]

Score	*n*	Oxidative DNA repair rates (SSBs/10^9 Da)
hOGG1 Ser326Cys, *XRCC1* Arg194Trp, Arg280His, Arg399Gln and *APE1* Asn148Glu		
0	14	1.05 ± 0.70
1	45	0.65 ± 0.59
2	58	0.72 ± 0.69
3	41	0.67 ± 0.69
4	17	0.49 ± 0.45
5	4	0.25 ± 0.22
$\chi^2 = 11.07$, $P = 0.050$		

The results are presented as mean ± SD. Comparisons were performed by Kruskal–Wallis test.
[a]Combinations were constructed on the base of an arbitrary score for variant alleles (See Materials and Methods for details). The higher score means the higher number of variant alleles in combined SNPs.

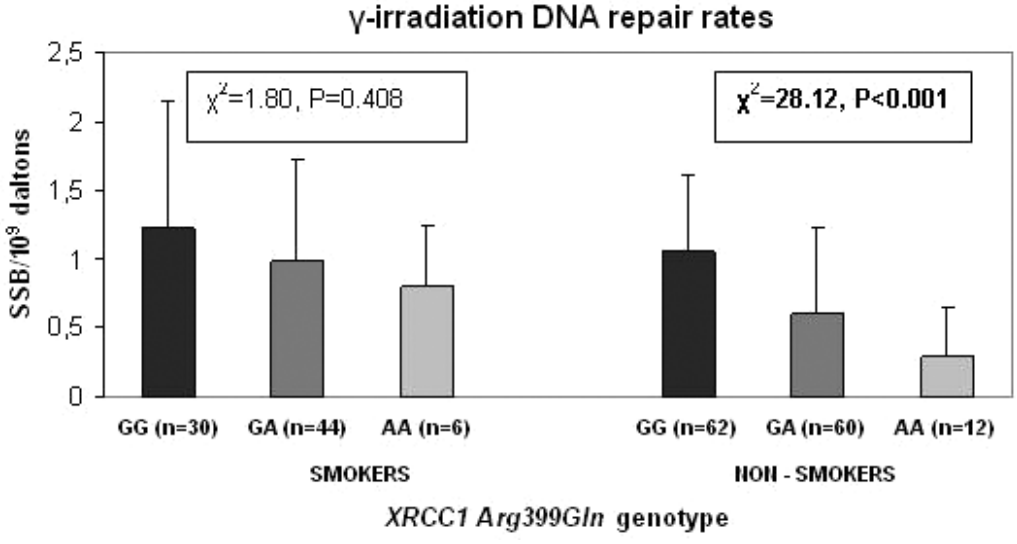

Fig. 1. The γ-irradiation DNA repair rates (expressed as SSB/10^9 Da) in individuals stratified for smoking habit (smokers $n = 80$, non-smokers $n = 134$) and for *XRCC1* Arg399Gln polymorphism. The results are presented as mean ± SD, statistical comparison was performed by Kruskal–Wallis test.

A significant decrease in irradiation-specific DNA repair rates was apparently associated with the binary combination of variant alleles in *XRCC1* Arg399Gln and *APE1* Asn148Glu polymorphisms. Although polymorphism in *APE1* Asn148Glu has no significant effect on irradiation-specific DNA repair, it seems to augment the effect exerted by *XRCC1* Arg399Gln polymorphism. An effect of various BER gene polymorphisms in combination on irradiation-specific DNA repair rates was also tested using an attributed score, reflecting a number of variant alleles in the particular combination, since all existing allele combinations could not be tested due to the low frequency of variant allele, particularly in *XRCC1* Arg194Trp and Arg280His. Apparently, the highest irradiation-specific DNA repair rates were associated with the lowest score, i.e. with the predominance of wild-type alleles in particular combinations. The results suggest that the main effect is due to the *XRCC1* Arg399Gln variant allele.

The capacity to repair oxidative DNA damage was 2-fold higher among individuals with the wild-type genotype (*CC*) in *hOGG1* Ser326Cys as compared to those with homozygous variant genotype. Although the larger functional studies also suggest reduced repair function with variant alleles in *hOGG1* (13,24), the evidence is generally inconclusive. On the other hand, variant *G* allele in *hOGG1* Ser326Cys was suggested to affect the glycosylase function due to the localization and phosphorylation status (15). Our data on the *hOGG1* Ser326Cys polymorphism and the capacity to repair oxidative DNA damage may provide more quantitative data on the decrease of oxidative damage repair in association with the variant allele in the above gene.

A significant decrease in the capacity to repair DNA oxidative damage was also associated with variant alleles in *hOGG1* Ser326Cys and *APE1* Asn148Glu polymorphisms, when this binary gene–gene interaction was investigated. Our data suggest that *APE1* Asn148Glu polymorphism contributes to highlight an effect of variant *G* allele in *hOGG1* Ser326Cys, although *APE1* Asn148Glu polymorphism itself did not influence the oxidative DNA damage repair capacity.

Binary combinations of polymorphisms in *hOGG1* Ser326Cys and *XRCC1* Arg194Trp, and Arg280His showed that the predominance of wild-type *C* allele in *hOGG1* Ser326Cys is associated with the higher capacity to repair DNA oxidative damage. The proper investigation of gene–gene interactions should be based on substantially larger population and the present data should be cautiously interpreted. Additionally, some other polymorphisms, such as those involved in nucleotide excision repair, may modulate levels of DNA damage as well as activity of OGG1 repair enzyme [higher activity was reported to be associated with the wild-type A allele in *XPA* gene, (25)].

By investigating simultaneous influence of genotypes in genes coding for BER enzymes and recorded confounders (age, sex, exposure status and smoking), irradiation-specific DNA repair rates were significantly affected by polymorphism in *XRCC1* Arg399Gln and by smoking. These data suggest the importance of gene–environment interactions and the research in this direction should be continued. Similarly, the capacity to repair DNA oxidative damage was significantly modulated by tentative exposure status and by *hOGG1* Ser326Cys polymorphism. A participation of environmental and occupational exposure factors on both irradiation-specific DNA repair rates as well as on the capacity to repair oxidative DNA damage has been reported earlier, suggesting that the particular DNA repair pathways may be induced by the exposure to xenobiotics (16,23).

An understanding of the relationships between DNA repair polymorphisms and corresponding functional reflections may contribute to the interpretation of results obtained from case–control association studies on various types of cancer. In order to clarify the roles of DNA repair polymorphisms and DNA repair capacities, as important susceptibility factors affecting the onset of cancer, both markers need to be analyzed first in general healthy population (background levels) and subsequently compared with those found in newly diagnosed, untreated cancer patients.

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Manuscript II

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Biomarkers of nucleic acid oxidation, polymorphism in, and expression of, hOGG1 gene in styrene-exposed workers

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Biomarkers of nucleic acid oxidation, polymorphism in, and expression of, *hOGG1* gene in styrene-exposed workers

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ABSTRACT

This study investigated nucleic acid oxidation associated with styrene exposure, mRNA expression levels of *hOGG1* gene and the role of the genetic polymorphism *Ser326Cys* of human 8-oxoguanine DNA N-glycosylase 1 (*hOGG1*) in 60 styrene-exposed workers and 50 unexposed clerks. Biomarkers of exposure (styrene in blood, mandelic and phenylglyoxylic acids and 4-vinylphenol in urine) and urinary biomarkers of nucleic acid oxidation, namely 8-oxo-7,8-dihydro-2′-deoxyguanosine (U-8-oxodGuo), 8-oxo-7,8-dihydroguanosine (U-8-oxoGuo) and 8-oxo-7,8-dihydroguanine (U-8-oxoGua) were determined by liquid chromatography–tandem mass spectrometry. The levels of 8-oxodGuo adduct and 2′-deoxyguanosine (dGuo) were measured by HPLC in DNA from white blood cells (WBC). Genomic DNA and RNA from blood samples were used to characterize the *Ser326Cys* polymorphism and the mRNA expression levels of the *hOGG1* gene, respectively, by PCR-based methods. Exposed workers showed lower values of 8-oxodGuo/10^5 dGuo ratio in WBC-DNA but higher concentrations of U-8-oxoGuo compared to controls ($p = 0.002$ and $p = 0.008$, respectively, *t*-test for independent samples). In the whole group, all urinary biomarkers of nucleic acid oxidation correlated with both the sum of mandelic and phenylglyoxylic acids (*rho* > 0.33, $p < 0.0001$) and 4-vinylphenol (*rho* > 0.29, $p < 0.001$), whereas 8-oxodGuo/10^5 dGuo in WBC showed a negative correlation with exposure parameters (*rho* < −0.24, $p < 0.02$). Subjects bearing the *hOGG1 Ser/Ser* genotype showed lower values of 8-oxodGuo/10^5 dGuo in WBC than those with at least one variant *Cys* allele (0.34 ± 0.16 *vs* 0.45 ± 0.21, $p = 0.008$). In the subgroup of *hOGG1 Ser/Ser* subjects, laminators showed lower levels of WBC 8-oxodGuo/10^5 dGuo ratio and significantly higher concentrations of U-8-oxoGua than controls ($p = 0.07$ and $p = 0.01$, respectively, *t*-test for independent samples). Interestingly, workers showed higher levels of *hOGG1* expression compared to controls ($p < 0.0005$). Styrene exposure seems to be associated with oxidation damage to nucleic acids, particularly to RNA and with an induction of the BER system.

1. Introduction

Styrene is a monomer widely used in the production of polymers, plastics and synthetic rubber. In humans, it is converted to styrene-(7,8)-oxide (7,8-SO) via the cytochrome P450 monooxygenase system (Nakajima et al., 1994). It is generally thought that most of styrene-induced genotoxicity is due to its electrophilic metabolite 7,8-SO, a highly reactive epoxide. Although styrene and 7,8-SO are known to induce both DNA adducts and DNA strand breaks in exposed workers, a recent re-evaluation of various genotoxic endpoints highlighted several inconsistencies in the overall current knowledge (Henderson and Speit, 2005; Vodicka et al., 2006). In

Abbreviations: hOGG1, human 8-oxoguanine DNA N-glycosylase 1; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; dGuo, 2′-deoxyguanosine; WBC, white blood cells; MTH1, mutT homolog-1; BER, base excision repair; 7,8-SO, styrene-(7,8)-oxide; MA, mandelic acid; PGA, phenylglyoxylic acid; 4-VP, 4-vinylphenol; LC–MS–MS, liquid chromatography–tandem mass spectrometry.

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addition, the outcomes from cancer epidemiological studies were also inconclusive (Kogevinas et al., 1993). However, the International Agency for Research on Cancer (IARC) classified styrene as possibly carcinogenic with limited evidence for carcinogenicity in humans and experimental animals, whereas 7,8-SO as probably carcinogenic to humans (IARC, 1994).

A hypothesis has been postulated that oxidative stress arising as an imbalance between oxidant and antioxidant molecules may also contribute to the genotoxic effects of styrene (Marczynski et al., 2000). *In vitro* studies have demonstrated that exposure to styrene or 7,8-SO induces increased lipid peroxidation and DNA oxidation as well as glutathione depletion (Chakrabarti et al., 1993; Vettori et al., 2005). The guanine moiety of nucleotides represents one of the main targets for hydroxyl radicals and, depending on the molecular context (2′-deoxyribonucleotides, ribonucleotides, DNA, RNA), oxidized guanine may undergo different repair pathways resulting in different extracellular reaction products (Lunec et al., 2002; Cooke et al., 2008). 8-Oxo-7,8-dihydroguanine (8-oxoGua) in DNA is selectively cleaved by specific glycosylases of the base excision repair (BER) system, including the polymorphic 8-oxoguanine DNA N-glycosylase 1 (hOGG1) (Cooke et al., 2003). Alternatively, oxidized guanine may be released from DNA as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) by an endonuclease-nucleotidase-based DNA repair system (Bessho et al., 1993). The same molecule is generated by the enzyme MTH1 (Tsuzuki et al., 2001) as product of repair of oxidized 2′-deoxyguanosine triphosphate in the cellular 2′-deoxyribonucleotide pool and by the nucleotide excision repair (NER) system, which releases oligonucleotides containing 8-oxodGuo (Patel et al., 2007). 8-Oxo-7,8-dihydroguanosine (8-oxoGuo) may originate from oxidized guanine in RNA, probably as a result of the turnover of the molecule, rather than as product of RNA repair mechanisms, that have not yet been well characterized (Nunomura et al., 2006). The turnover or repair of RNA may be responsible also for the generation of extracellular 8-oxoGua (Evans and Cooke, 2004).

In humans, 8-oxodGuo has been extensively studied either in DNA isolated from white blood cells (WBC) or as free urinary deoxynucleoside, although some inconclusive results have been reported in the field of occupational end environmental exposures (Pilger and Rudiger, 2006). More recently, the advent of liquid chromatography–tandem mass spectrometry (LC–MS–MS) has enabled the determination of oxidatively modified guanine derivatives in urine samples (U-), such as U-8-oxodGuo, U-8-oxoGuo, and U-8-oxoGua (Weimann et al., 2002). Such an approach limits the risk of artifactual oxidation during pre-analytical phases, owing to the minimal sample manipulation prior to injection. The combined evaluation of oxidized guanine derivatives both in DNA from white blood cells (WBC-DNA) and in urine may allow a better understanding of genotoxic mechanisms at the molecular level. DNA oxidation may be modulated by germ line variants in DNA repair genes, the most prominent being hOGG1. For this enzyme, a functional genetic polymorphism is known (*Ser326Cys*) and the variant allele distributes in Caucasian with a prevalence of about 20% (Marchand et al., 2002). Although the association between *hOGG1* genotype and the enzyme activity of OGG1 has not been definitely proven so far (Weiss et al., 2005), experimental investigations demonstrate that the hOGG1 Cys326 isozyme has impaired function (by about 2-fold) compared to the Ser326 isoform (Luna et al., 2005; Bravard et al., 2009). In agreement with these data, a recent epidemiological study (Vodicka et al., 2007) has shown that subjects with the *hOGG1 Cys/Cys* genotype exhibit a 50% lower DNA repair capacity when compared to *hOGG1 Ser/Ser* subjects.

The present study was carried out to investigate the levels of oxidized guanine derivatives in a group of styrene-exposed workers with accurately characterized internal dose levels, and an unexposed control group. As an additional aim, we evaluated the modulating role of both gene expression and genetic polymorphism of the *hOGG1* gene on oxidatively generated DNA damage associated with styrene exposure. In particular, we evaluated the relationships between the levels of the oxidized guanine in WBC-DNA (determined as 8-oxodGuo/10^5 dGuo) that is the relevant substrate for hOGG1 activity and the urinary concentrations of the reaction product 8-oxoGua in subjects classified by the *hOGG1 Ser326Cys* polymorphism.

Table 1
Characteristics of the studied population and characterization of styrene exposure. Exposure biomarkers are reported as median and range.

	Controls ($n = 50$)	Exposed ($n = 60$)
Sex (male/female)	39/11	42/18
No. of current/never-smokers	13/37	26/34
Age (years)	40.0 ± 12.1 (26–62)	37.6 ± 11.1 (21–64)
Years of employment	–	4.0 ± 3.4 (1–14)
Styrene air, mg/m^3	n.d.	107.4 ± 66.7
Blood styrene, mg/L	0.20 (n.d. to 0.43)	1.20 (n.d. to 3.94)
MA + PGA, mg/g creatinine	0.47 (0.17–3.24)	286.7 (4.16–2022)
4-VP, mg/g creatinine	0.19 (0.01–4.08)	3.39 (0.19–22.6)

Note: 1 ppm of styrene is equal to 4.25 mg/m^3. MA + PGA: mandelic acid + phenylglyoxylic acid; 4-VP: 4-vinylphenol; n.d.: not detectable.

2. Materials and methods

2.1. Subjects and sampling

Sixty styrene-exposed workers employed in two plastics lamination plants in the same geographical area and 50 unexposed clerks volunteered to participate in the study. Confounding factors, like X-rays, medical drug treatment, dietary and lifestyle were carefully controlled by detailed questionnaire. The study was conducted on healthy individuals and exclusion criteria comprised a recent exposure to X-rays, current drug use or viral infections experienced in the last 3 months. The main characteristics of the studied population are reported in Table 1. The local Ethical Committee approved the study protocol and the participating subjects provided their written informed consent. The sampling of biological material was carried out according to the Helsinki Declaration (WHO, 1964).

Spot urine samples (50 mL) were collected at the end of the shift, divided into two aliquots and frozen at −20 °C until analysis. Blood samples (40 mL) were collected from all subjects in the middle of the work shift (ensuring saturation) on the same day as collection of urine samples.

2.2. Chemicals

Styrene (purity 99%), DL-mandelic acid (MA, 98%), phenylglyoxylic acid (PGA, 98%), 2′-deoxyguanosine (dGuo, 99–100%), and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo, 98%) were purchased by Sigma–Aldrich (Taufkirchen, Germany and Milan, Italy). 8-Oxo-7,8-dihydroguanosine (8-oxoGuo, 98%) and 8-oxo-7,8-dihydroguanine (8-oxoGua, 90%) were from Cayman (MI, USA). Isotopically labeled compounds used as either internal standards (ISs), *i.e.* [$^{13}C_1$, $^{15}N_2$]8-oxoGua ($^{13}C_1$ 98%, $^{15}N_2$ 98%) and [$^{15}N_5$]Guo (96–98%, used as IS for 8-oxoGuo), or for the synthesis of ISs, *i.e.* [$^{15}N_5$]dGuo (96–98%), were obtained from Cambridge Isotope Laboratories Inc. (MA, USA). [$^{15}N_5$]8-oxodGuo had been synthesized from [$^{15}N_5$]dGuo according to Hu et al. (2004), with minor modifications. All standards were used without further purification.

2.3. Styrene exposure at the workplace

The concentration of airborne styrene at the workplace was determined by personal dosimeters on the day of the sampling, as previously described (Vodicka et al., 1995). Results are summarized in Table 1.

2.4. Exposure biomarkers

Styrene in the blood was determined as previously described (Vodicka et al., 1995, 2001). Styrene metabolites, namely MA, PGA, and 4-VP were determined by LC–MS–MS as previously described (Manini et al., 2002). Concentrations of urinary metabolites were expressed as a function of creatinine concentration (mg/g creat.), measured by the method of Jaffe (Kroll et al., 1986). Sample with creatinine concentrations lower than 0.3 g/L or higher than 3.0 g/L were excluded from statistical analysis according to the American Conference of Governmental Industrial Hygienists recommendation (ACGIH, 2004). For quantitative analyses, calibrations were performed in a matrix by spiking pooled urine samples from non-exposed subjects with appropriate standard mixtures. The concentrations of styrene in blood and

styrene metabolites in styrene-exposed workers and unexposed clerks are reported in Table 1.

2.5. Urinary biomarkers of nucleic acid oxidation

Urinary levels of 8-oxodGuo, 8-oxoGuo and 8-oxoGua, were determined by isotopic dilution LC–MS–MS, by adapting the method proposed by Weimann et al. (2002). Filtered samples (0.2 mL) were added with an equal volume of IS mixture containing 2.5 μg/L of [$^{15}N_5$]8-oxodGuo, 7.5 μg/L of [$^{15}N_5$]Guo and 75 μg/L of [$^{13}C_1$,$^{15}N_2$]8-oxoGua dissolved in 100 mM lithium acetate (pH 6.4). Then, samples were kept at 37 °C for 10 min, vortexed, centrifuged (10 min at 10,000 × *g*) and injected (30 μL). Separation of urinary 8-oxodGuo, 8-oxoGuo and 8-oxoGua was performed on an Atlantis®dC$_{18}$ column (100 mm × 2.0 mm i.d., 3 μm; Waters, Milford, MA, USA) using variable proportions of 10 mM aqueous formic acid (pH 3.75) and methanol at a flow-rate of 0.2 mL/min. After the column, a flow of 0.07 mL/min of methanol was added to the chromatographic flow to improve the ionization efficiency. The analytes and the ISs were ionized in positive ion mode and the detection was obtained in *selected-reaction monitoring* mode by following the transitions: *m/z* 284 → 168 and *m/z* 289 → 173 for 8-oxodGuo and its IS [$^{15}N_5$]8-oxodGuo; *m/z* 300 → 168 and *m/z* 289 → 157 for 8-oxoGuo and its IS [$^{15}N_5$]Guo; and *m/z* 168 → 140 and *m/z* 171 → 142 for 8-oxoGua and its IS [$^{13}C_1$, $^{15}N_2$]8-oxoGua. For quantitative analysis, working calibrations were obtained by spiking pooled urines with standard solutions in the concentrations ranges 0.25–25 μg/L for 8-oxodGuo, 0.75–75 μg/L for 8-oxoGuo and 7.5–750 μg/L for 8-oxoGua. For each analyte, calibration curves were constructed by linear regression analysis of the analyte-to-IS area ratio *vs* the known concentration of analytes injected ($r^2 > 0.998$). The limits of quantification (LoQs) were 0.2, 0.3 and 1.0 μg/L for 8-oxodGuo, 8-oxoGuo and 8-oxoGua, respectively. Intra- and inter-day precision ranged between 2.5% and 6.8% for all analytes. Results were expressed as a function of creatinine concentration (μg/g creat.). All analytical determinations of urinary biomarkers were performed on a PE-Sciex API 365 triple-quadrupole mass spectrometer (Applied Biosystems, Thornhill, Canada) equipped with a TurboIonSpray™ interface.

2.6. 8-OxodGuo adduct in WBC-DNA

Blood samples (9 mL) were collected in EDTA-treated tubes, immediately frozen at −20 °C and used for 8-oxodGuo adduct determination. White blood cells (WBC) were collected by centrifugation with 35 mL of 0.9% NH_4Cl, 10 mM Na_2EDTA similar to the procedure of Fichtinger-Schepman et al. (1987). DNA was extracted with chloroform following operation of Dahlhaus and Appel (1993) and recommendations of ESCODD (2002) to avoid artefacts during DNA preparation. 8-OxodGuo adduct isolation was carried out by previously published procedure (Marczynski et al., 2002). For the analysis of nucleosides in WBC-DNA, a Shimadzu HPLC/UV apparatus, connected to a Coulochem II (model 5200) electrochemical detector (ESA, Chelmosford, MA, USA), was used.

2.7. hOGG1 expression levels

Peripheral blood lymphocytes were isolated from 10 mL of peripheral blood and mRNA was immediately extracted by TRIzol®, according to the producer's procedure (Invitrogen, Paisley, UK). RNA quality and quantity was checked by UV VIS spectrophotometry on Carry 300 (Varian, Palo Alto, CA, USA) and horizontal agarose gel electrophoresis. cDNA was synthesized from 0.5–1 μg of total mRNA using First strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania). As a negative control, we used the same sample, but reverse transcriptase was omitted from the mixture. Quality of cDNA was confirmed by PCR amplification of fragment from the control gene ubiquitin C (Soucek et al., 2005). The PCR product (190 bp) in the negative sample indicates whether cDNA was contaminated by genomic DNA (1009 bp).

Expression of *hOGG1* gene was determined with a 7500 Real Time PCR System (Applied Biosystems, Foster City, USA) as absolute quantification, using TaqMan Gene Expression Assays 4331182 (Hs00213454_m1/hOGG1/, Applied Biosystems). As standards for absolute quantification of gene expression, we used bacterial plasmids containing coding sequences of *hOGG1* and *GAPDH*, prepared by the GatewayTM cloning technology (Invitrogen, Paisley, UK) as previously described (Baranová et al., 2005; Suzuki et al., 2005). The expression of *hOGG1* was normalized to the expression of *GAPDH* (TaqMan Endogenous control Human GAPDH 4333764; Diodovich et al., 2004) and expressed in arbitrary units.

2.8. hOGG1 genotype analysis

Genomic DNA was extracted from 3 mL of peripheral whole blood by a commercial kit (PureGene, GENTRA SYSTEMS, Minneapolis, MN). *hOGG1* Ser326Cys polymorphism (rs 1052133) was characterized according to a previously published PCR-RFLP method (Wikman et al., 2000). In the whole study group, the following figures were observed: *hOGG1 Ser/Ser* genotype was present in 69 individuals (65%), *Ser/Cys* in 30 (28%), and *Cys/Cys* in 7 (7%). Similar genotype frequencies were observed among workers ($n = 59$), *i.e.* 39 (66%) individuals with *Ser/Ser*, 18 (31%) with *Ser/Cys*, and 2 (3%) with *Cys/Cys* genotypes, and controls ($n = 47$), *i.e.* 30 (64%) with the *Ser/Ser*, 12 (26%) with the *Ser/Cys* and 5 (10%) with the *Cys/Cys* genotypes. These frequencies were consistent with the expectations for the Hardy–Weinberg equilibrium. Determination of *hOGG1* genotypes was confirmed by random re-genotyping, using the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, USA).

2.9. Statistical analyses

Statistical analyses were carried out by the SPSS software (version 14.0 for Windows®, Chicago, IL). Although all analytical determinations were above the corresponding limit of detections, valid samples (having urinary creatinine between 0.3 and 3.0 g/L) amounted to 104 and complete information including urinary exposure indices, biomarkers of nucleic acid oxidation, and *hOGG1* genotypes was available for 96 subjects. The normality of the distribution was assessed by the one-sample Kolmogorov–Smirnov test. Parametric statistical tests were applied to log-transformed values ensuring a normal distribution of variables. Biomarkers of nucleic acid oxidation were normally distributed, 4-VP followed a log-normal distribution, whereas MA + PGA values were not distributed normally or log-normally. Differences between the exposed and control groups were assessed using the *t*-test for independent samples. Pearson's *r* was used to assess the correlation between variables. Non-parametric tests were also applied, when necessary (Mann–Whitney *U*-test and Spearman's *rho*). The significance level for all tests was $p \leq 0.05$ (two-tailed). Multiple linear regression analysis models were used to assess the contribution of sex, age, smoking habits, urinary creatinine concentration and exposure to styrene (as MA + PGA concentrations) to the variability of biomarkers of nucleic acid oxidation. Stepwise regression analyses were run using a significance level 0.05 for entry and 0.10 for variables' removal from the model. Due to the limited sample size, in statistical analyses the *hOGG1* genotypes were collapsed into two groups (according to a recessive model of inheritance), one represented by the homozygous wild-type (*Ser/Ser*) subjects and the other including people bearing at least one *Cys* allele (*Ser/Cys* and *Cys/Cys*).

3. Results

3.1. Internal dose markers

The concentrations of internal dose markers, *i.e.* styrene in blood and urinary metabolites (MA, PGA, and 4-VP) in subjects classified according to exposure status (workers *vs* controls) are summarized in Table 1. As biomarkers were not normally distributed in the whole study group, their concentrations are reported as medians and ranges. MA + PGA concentrations in controls were lower than the 95th percentile of the frequency distribution of the biomarker in the general unexposed population, *i.e.* 3.5 mg/g creat. (Manini et al., 2004). Conversely, all workers showed MA + PGA concentrations higher than such value. The distributions of internal dose biomarkers in controls and workers did not overlap.

In the whole study group, markers of exposure significantly correlated to each other ($p < 0.0005$), the Spearman's correlation coefficients being *rho* = 0.64 between styrene in blood and MA + PGA, *rho* = 0.53 between styrene in blood and 4-VP, and *rho* = 0.90 between MA + PGA and 4-VP. All these correlations were observed in the worker group (*rho* = 0.49, 0.38 and 0.94, respectively, $p \leq 0.0005$ for all) whereas among controls only urinary biomarkers were significantly correlated (*rho* = 0.65, $p < 0.0005$).

3.2. Biomarkers of nucleic acid oxidation

Urinary levels of oxidatively modified guanine derivatives significantly correlated to each other. The Pearson's correlation coefficients were $r = 0.86$ between U-8-oxodGuo and U-8-oxoGuo,

Table 2
Biomarkers of nucleic acid oxidation in controls ($n = 50$) and workers ($n = 60$). Urinary biomarkers have been expressed as a function of creatinine concentration. Values are expressed as mean ± S.D.

Biomarker	Controls ($n = 50$)	Exposed ($n = 60$)	*p*
WBC 8-oxodGuo/10^5 dGuo	0.46 ± 0.23	0.33 ± 0.13	0.002
U-8-oxoGua (μg/g creat.)	16.92 ± 5.90	18.73 ± 8.35	0.739
U-8-oxodGuo (μg/g creat.)	3.81 ± 1.49	3.91 ± 1.38	0.243
U-8-oxoGuo (μg/g creat.)	4.55 ± 1.53	5.58 ± 2.03	0.008

U-: urinary; 8-oxodGuo: 8-oxo-7,8-dihydro-2′-deoxyguanosine; dGuo: 2′-deoxyguanosine; 8-oxoGua: 8-oxo-7,8-dihydroguanine; 8-oxoGuo: 8-oxo-7,8-dihydroguanosine.

Table 3
Predictors of the urinary excretion of biomarkers of nucleic acid oxidation (set as dependent variable) according to a stepwise multiple linear regression model: (U-biomarker) = constant + (U-creatinine) × β_1 + (U-MA + PGA) × β_2 + (Age) × β_3. Values of constant and β coefficient, with S.E., partial r^2 (r_p^2) and significance (p) for each term are given. The adjusted r^2 (r_{adj}^2) and significance (p) for the whole model are reported in the last row. The significance level was 0.05 for entry and 0.10 for removal from the model.

	U-8-oxodGuo			U-8-oxoGuo			U-8-oxoGua		
	β (S.E.)	r_p^2	p	β (S.E.)	r_p^2	p	β (S.E.)	r_p^2	p
Constant	0.32 (0.24)	–	0.179	−1.19 (0.62)	–	0.057	3.81 (1.12)	–	0.001
U-creatinine	3.04 (0.23)	0.642	<0.0005	4.35 (0.30)	0.674	<0.0005	12.63 (1.08)	0.578	< 0.0005
U-MA + PGA	0.0012 (0.0004)	0.026	0.006	0.0019 (0.0006)	0.029	0.001	–	–	–
Age	–	–	–	0.037 (0.014)	0.020	0.01	–	–	–
Whole model r_{adj}^2, p		0.662	<0.0005		0.714	<0.0005		0.573	<0.0005

U-: urinary; 8-oxodGuo: 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGuo: 8-oxo-7,8-dihydroguanosine; 8-oxoGua: 8-oxo-7,8-dihydroguanine; MA + PGA: mandelic acid + phenylglyoxylic acid.

$r = 0.65$ between U-8-oxodGuo and U-8-oxoGua, and $r = 0.75$ between U-8-oxoGuo and U-8-oxoGua ($p < 0.0005$). These correlations were also observed within the subgroups of workers and controls (data not shown). Urinary biomarkers of nucleic acid oxidation were strongly correlated with urine creatinine, the Pearson's correlation coefficient ranging between 0.76 and 0.82 ($p < 0.0005$).

No correlation was detected between WBC 8-oxodGuo/10^5 dGuo and urinary oxidized guanine derivatives. Moderate, but significant, correlations were observed among biomarkers of exposure and biomarkers of nucleic acid oxidation in the whole study group. WBC 8-oxodG/10^5 dGuo was inversely correlated with both MA + PGA (*rho* = −0.24, $p = 0.019$) and 4-VP (*rho* = −0.25, $p = 0.016$), whereas U-8-oxodGuo, U-8-oxoGuo and U-8-oxoGua were positively correlated with both MA + PGA (*rho* = 0.33, 0.41 and 0.34, respectively, $p < 0.0001$) and 4-VP (*rho* = 0.32, 0.40 and 0.29, respectively, $p < 0.001$). Age did not modulate significantly any of the above markers (data not shown).

The effect of exposure and potentially confounding factors, such as sex and smoking habits, on biomarkers of nucleic acid oxidation was initially assessed by univariate analyses. The WBC 8-oxodGuo/10^5 dGuo ratio was significantly lower in the exposed group than in the controls ($p = 0.002$, *t*-test for independent samples, Table 2). As urinary creatinine was not significantly different in workers and controls (0.96 ± 0.61 g/L *vs* 0.79 ± 0.48 g/L, *n.s.*) and according to our yet unpublished results showing that urinary biomarkers of nucleic acid oxidation and urinary creatinine showed similar kinetics of excretion, we normalized oxidation markers by creatinine concentration. Workers showed significantly higher U-8-oxoGuo levels compared to controls ($p = 0.008$), whereas no significant difference was observed for U-8-oxoGua and U-8-oxodGuo (Table 2). No differences either in the levels of oxidation damage biomarkers or in the concentration of urinary creatinine were found in the group stratified by sex (data not shown). Conversely, in this sample, smokers showed higher levels of urinary creatinine than non-smokers (1.04 ± 0.54 g/L *vs* 0.79 ± 0.54 g/L, $p = 0.028$).

Multiple regression models run to assess the role of styrene exposure (as MA + PGA) and other predictors (sex, age, smoking habits and urinary creatinine) on urinary biomarkers of nucleic acid oxidation substantially confirmed the findings of univariate analyses. The results of stepwise models are summarized in Table 3, where partial r^2 values are reported to evaluate the individual contribution of each predictor to the overall variance. Creatinine alone accounted for more than 58% of variance of all biomarkers (with partial r^2 ranging between 0.58 and 0.67, $p < 0.0001$ for all). Significant relationships between urinary biomarkers of nucleic acid oxidation and exposure biomarkers were observed for U-8-oxodGuo ($p = 0.006$) and U-8-oxoGuo ($p = 0.001$), exposure accounting for 2.6 and 2.9% of variance, respectively. In any case, the smoking status and gender did not significantly affect the concentration of urinary biomarkers. Age significantly influenced the levels of U-8-oxoGuo only ($p = 0.01$).

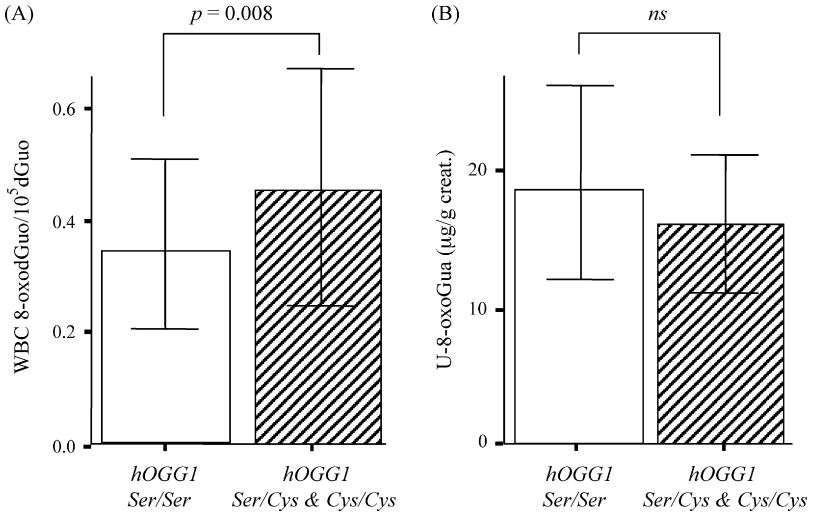

Fig. 1. Comparison between (A) the levels of the WBC 8-oxodGuo/10^5 dGuo ratio and (B) the concentrations of U-8-oxoGua in subjects stratified according to the *hOGG1* polymorphism (*t*-test for independent samples). Data are expressed as mean ± S.D. *hOGG1* genotypes were collapsed into two groups (recessive model): the homozygous wild-type (*Ser/Ser*) genotype ($n = 64$) and genotypes including at least one *Cys* allele (*Ser/Cys* and *Cys/Cys*, $n = 35$).

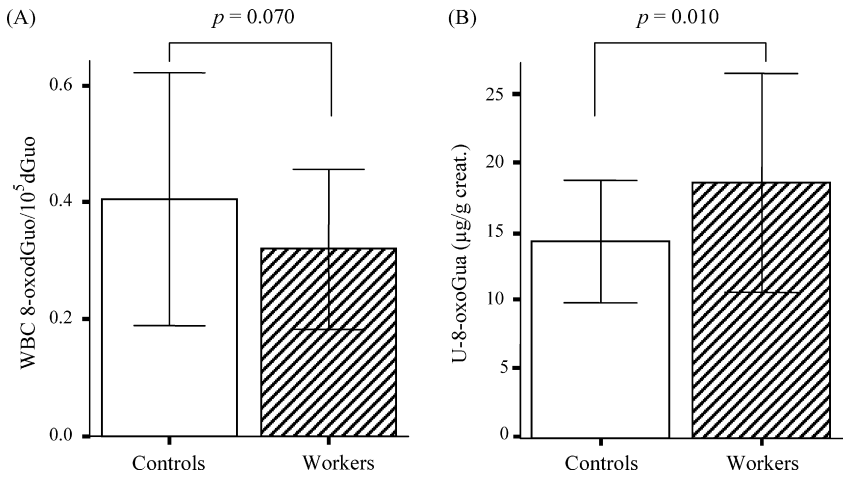

Fig. 2. Comparison between (A) WBC 8-oxodGuo/10^5 dGuo and (B) U-8-oxoGua in controls and styrene-exposed workers. Only subjects bearing the *hOGG1 Ser/Ser* genotype were considered (*t*-test for independent samples). Data are expressed as mean ± S.D.

3.3. hOGG1 Ser326Cys polymorphism and expression levels of hOGG1

In the whole sample, subjects bearing the *Ser/Ser* genotype showed significantly lower levels of WBC 8-oxodGuo/10^5 dGuo (about 26%, on average) than those carrying the variant *Cys* allele (0.34 ± 0.16 *vs* 0.46 ± 0.21, $p = 0.008$, Fig. 1A). A similar trend was observed among workers (0.31 ± 0.11 *vs* 0.38 ± 0.17, $p = 0.09$), but the difference was not significant. Fig. 1B shows that in the whole sample, individuals with the wild-type *Ser/Ser* genotype tended to excrete higher concentration of U-8-oxoGua than those carrying variant *Cys* allele (18.18 ± 7.91 μg/g creat. *vs* 16.76 ± 5.50 μg/g creat., *n.s.*). Among workers with homozygous wild-type genotype (*hOGG1 Ser/Ser*), significantly lower 8-oxodGuo/10^5 dGuo levels (0.31 ± 0.11 *vs* 0.40 ± 0.22, $p = 0.05$) and significantly higher concentrations of U-8-oxoGua than in controls (18.73 ± 8.01 μg/g creat. *vs* 14.4 ± 4.48 μg/g creat., $p = 0.01$) were observed (Fig. 2).

Styrene-exposed workers also showed increased levels of *hOGG1* expression compared to controls (0.0032 ± 0.0015 a.u. *vs* 0.0020 ± 0.0009 a.u., $p < 0.0005$, *t*-test for independent samples). Moreover, females showed higher levels of *hOGG1* expression compared to males (0.0032 ± 0.0013 a.u. *vs* 0.0025 ± 0.0014 a.u., $p = 0.022$).

The *hOGG1 Ser326Cys* polymorphism did not significantly affect the levels of *hOGG1* expression. No differences were observed between subjects bearing the *Ser/Ser* genotype and those carrying variant *Cys* allele, either in the whole sample (0.0027 ± 0.0015 a.u. *vs* 0.0026 ± 0.0013 a.u.), or in subgroups of styrene-exposed workers (0.0033 ± 0.0017 a.u. *vs* 0.0032 ± 0.0014 a.u.) and controls (0.0020 ± 0.0010 a.u. *vs* 0.0018 ± 0.0007 a.u.).

4. Discussion

This is the first communication reporting the application of a panel of blood DNA and urinary biomarkers of nucleic acid oxidation to characterize the extent oxidative stress in workers exposed to styrene. In the present study, urinary biomarkers of oxidation damage have been evaluated by a method based on isotopic dilution LC–MS–MS, which limits sample manipulation prior to analysis and further reduces the risk of guanine oxidation during preparative steps. It should be noted that the sources and the meaning of urinary nucleobases, 2′-deoxyribonucleosides and ribonucleosides reflecting oxidation damage to nucleic acids have not been fully characterized (Cooke et al., 2008). Whereas oxidation of 8-position of guanine in DNA leads to a unique product, determined as 8-oxodGuo adduct in WBC-DNA, several urinary biomarkers may be generated, depending on the localization of the guanine residue (DNA, RNA or the nucleotide pool) and the specificity and efficiency of the involved repair systems.

As many recent studies pointed out, the net oxidative damage to DNA results from the three-way balance between oxidizing species, antioxidants and DNA repair (Friedberg, 2001). Thus, the actual levels of 8-oxodGuo/10^5 dGuo in DNA from WBC are indicative of not yet repaired oxidation damage to DNA. The present study shows that styrene-exposed workers have significantly lower amount of oxidation damage in WBC-DNA compared to controls. An opposite result was previously reported in a small study, where higher levels of WBC 8-oxodGuo/10^5 dGuo were found in styrene-exposed boat builders (Marczynski et al., 1997). Several aspects may account for the discrepancy, *e.g.* different exposure conditions, co-exposure to other oxidant compounds, like diisocyanates, and the limited group of 17 workers, precluding any robust statistical evaluation. In our study, significantly higher *hOGG1* transcript levels were observed among workers, suggesting an induction *of hOGG1* gene by styrene exposure, that would be fully consistent with the observed levels of WBC 8-oxodGuo/10^5 dGuo.

Despite the debate about the measurement and the meaning of urinary oxidized guanine derivative species in urine (Cooke et al., 2008), we know that U-8-oxoGua originates, at least in part, from the glycosylase activity (BER) on oxidized guanine residues of DNA, but a major source is likely to be RNA, too. On the other hand, urinary concentrations of 8-oxodGuo may reflect either the repair (by MTH1 or NUDT1) of oxidized guanine triphosphate in the nucleotide pool or the repair of 8-oxodGuo from DNA (by an endonuclease/nucleotidase system) or even repair by NER (Patel et al., 2007). It is reasonable to assume that U-8-oxoGuo originates from oxidation of RNA (Evans and Cooke, 2004). In our study, the urinary levels of U-8-oxoGuo were significantly increased in workers compared to controls and significantly associated with styrene exposure, as a consequence of cytoplasmatic oxidative stress. RNA is single-stranded and its bases are protected neither by hydrogen bonds nor by structural proteins and may be more susceptible to oxidative insults than DNA (Nunomura et al., 2006). Moreover, the localization of styrene metabolizing CYP in the endoplasmatic reticulum that are an important source of ROS, is compatible with secondary oxidation of RNA molecules that are located in the neighbouring cytoplasm. Despite urinary concentrations of 8-oxodGuo were comparable in controls and exposed, they were significantly associated with styrene exposure in multivariate models. In the case of U-8-oxoGua, the product of hOGG1 activity, we did not

observe any difference between exposed workers and controls and any association with styrene exposure, probably due to the individual genetic background, represented here by the *hOGG1* Ser326Cys polymorphism. In addition, RNA could be an additional source of extracellular 8-oxoGua.

Mice knocked out at the *OGG1* locus showed a 26% reduction in the levels of U-8-oxoGua compared to the wild-type strain (Rozalski et al., 2005). In agreement with above study, we found reduced WBC 8-oxodGuo/10^5 dGuo levels in subjects bearing the *Ser/Ser* genotype, compared to those carrying the variant *Cys* allele. In the same subjects, we observed a complementary, though not significant, increase in mean U-8-oxoGua excretion in *Ser/Ser* subjects (Fig. 1). The effect of *hOGG1* polymorphism seems to be more specific for the substrate (8-oxodGuo/10^5 dGuo in WBC-DNA) than for the reaction product (U-8-oxoGua), which may derive from alternative pathways. The *hOGG1* Ser326Cys polymorphism did not affect the levels of *hOGG1* mRNA expression and this is consistent with the functional nature of the polymorphism that affects the enzyme activity by the Ser326Cys change in the primary protein structure (Bravard et al., 2009).

To evaluate the interaction between *hOGG1* polymorphism and styrene exposure, we limited the analysis to individuals with the wild-type *Ser/Ser* genotype. In this subgroup of subjects, workers showed lower levels of the substrate 8-oxodGuo/10^5 dGuo in WBC and significantly higher concentrations of reaction product U-8-oxoGua than controls (Fig. 2), suggesting that styrene exposure may induce BER enzymes. In agreement with this, the lower levels of WBC 8-oxodGuo/10^5 dGuo in exposed subjects are accompanied with the higher *hOGG1* expression levels, providing pilot mechanistic data.

We observed a strong correlation between urinary concentrations of oxidatively modified guanine derivatives and creatinine (Pearson's $r > 0.76$, $p < 0.0005$). This result has never been reported before. It is recommended that, in future studies, possible differences in urinary creatinine should be checked in the investigated groups and results should be confirmed by multivariate models including urinary creatinine concentration as covariate or independent variable (Barr et al., 2005). Although urinary creatinine alone accounted for more than 58% of total biomarker variance, regression analysis revealed that about 2–3% of the variability of U-8-oxodGuo and U-8-oxoGuo could be ascribed to styrene exposure (Table 3). However, our data should be considered cautiously, due to the limited sample size of the study population. Comparison of present results with those published earlier by Marczynski et al. (1997) and Vodicka et al. (2004) shows the importance of a well-matched control group without any exposure to styrene.

The present study seems to indicate that styrene exposure is associated with oxidation damage to nucleic acids, particularly to RNA, and with an induction of the BER system, as suggested by increased *hOGG1* expression levels in exposed workers and by the complementary distribution of DNA damage in blood and repaired DNA damage in urine in subjects bearing the *hOGG1 Ser/Ser* genotype (Fig. 2). Within the exposure range explored in this study, styrene-induced oxidative damage appeared to be counterbalanced by DNA repair capacity. About 75% of workers showed urinary concentrations of MA + PGA lower than 600 mg/g creat., a value corresponding to the Biologischer Arbeitsstoff-Toleranz-Wert (BAT, biological tolerance values) proposed by the German Deutsche Forschungsgemainschaft (DFG). This value corresponds to the maximum workplace concentration (MAK) of airborne styrene that do not have an impact on health even for long-term exposure, *i.e.* 20 ppm (DFG, 2007). For these workers, the BER mechanisms appeared to be effective in removing oxidation damage from WBC DNA. Conversely, Fracasso et al. (2009) recently reported a significant decrease of DNA repair activity in workers exposed to about 47 ppm styrene compared to controls. We speculate that styrene exposure could result in the induction of DNA oxo-repair enzymes, though this mechanism could be overwhelmed by higher dose levels. Interestingly, the threshold for the onset of clastogenic genotoxic effects due to styrene exposure has been identified at 125 mg/m^3 (or 30 ppm) (Nestmann et al., 2005). Below this threshold, the induction of DNA repair enzyme would be sufficient to balance the DNA damage, as shown by the results of our study. Above this threshold, a reduced DNA repair capacity (Fracasso et al., 2009) could lead to the persistence of reactive styrene metabolites responsible for clastogenic effects of styrene in humans.

The present data are consistent with our previous findings, indicating that both single strand break repair and removal of oxidized guanine are induced by styrene exposure, as assessed by methods based on the comet assay (Vodicka et al., 2004). In this study, we focused on accurate analyses of specific oxo-adducts in various biological matrices, both revisiting well-know biomarkers of nucleic acid oxidation, like 8-oxodGuo/10^5 dGuo in WBC-DNA and U-8-oxodGuo, and investigating less frequently considered biomarkers, like U-8-oxoGuo and U-8-oxoGua.

5. Conclusion

This study demonstrated a significant relationship between styrene exposure and biomarkers of nucleic acid oxidation. Among urinary biomarkers, 8-oxoGuo (specific for RNA oxidation) and 8-oxodGuo (biomarker of DNA oxidation) were the most susceptible to oxidative damage secondary to styrene exposure, whereas 8-oxoGua (the main product of DNA oxidation) was increased only in exposed subjects bearing the *hOGG1 Ser/Ser* genotype. The *hOGG1* Ser326Cys polymorphism could display some interference as an effect modifier *in vivo*. Finally, at the observed exposure levels, styrene was able to induce the expression and the activity of *hOGG1*.

Conflict of interest statement

Authors declare that they have no competing interests.

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Manuscript III

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Modulation of DNA repair capacity and mRNA expression levels of XRCC1, hOGG1 and XPC genes in styrene-exposed workers

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ABSTRACT

Decreased levels of single-strand breaks in DNA (SSBs), reflecting DNA damage, have previously been observed with increased styrene exposure in contrast to a dose-dependent increase in the base-excision repair capacity. To clarify further the above aspects, we have investigated the associations between SSBs, micronuclei, DNA repair capacity and mRNA expression in XRCC1, hOGG1 and XPC genes on 71 styrene-exposed and 51 control individuals. Styrene concentrations at workplace and in blood characterized occupational exposure. The workers were divided into low (below 50 mg/m^3) and high (above 50 mg/m^3) styrene exposure groups. DNA damage and DNA repair capacity were analyzed in peripheral blood lymphocytes by Comet assay. The mRNA expression levels were determined by qPCR. A significant negative correlation was observed between SSBs and styrene concentration at workplace ($R=-0.38$, $p=0.001$); SSBs were also significantly higher in men ($p=0.001$). The capacity to repair irradiation-induced DNA damage was the highest in the low exposure group (1.34 ± 1.00 SSB/10^9 Da), followed by high exposure group (0.72 ± 0.81 SSB/10^9 Da) and controls (0.65 ± 0.82 SSB/10^9 Da). The mRNA expression levels of *XRCC1*, *hOGG1* and *XPC* negatively correlated with styrene concentrations in blood and at workplace ($p<0.001$) and positively with SSBs ($p<0.001$). Micronuclei were not affected by styrene exposure, but were higher in older persons and in women ($p<0.001$). In this study, we did not confirm previous findings on an increased DNA repair response to styrene-induced genotoxicity. However, negative correlations of SSBs and mRNA expression levels of *XRCC1*, *hOGG1* and *XPC* with styrene exposure warrant further highly-targeted study.

Introduction

Styrene is a monomer extensively used in chemical industries for the production of various plastics and polyester resins and also represents an environmental contaminant (IARC, 2002). Styrene and its primary reactive metabolite styrene-7,8-oxide (SO), the substrate suggested to impart main genotoxic effect, have been classified as possible (2B) and probable (2A) human carcinogens (IARC, 1994). Styrene reportedly induces a wide spectrum of DNA adducts (Vodicka et al., 2002) that may be repaired via different repair pathways, with assumption of base-excision repair (BER) being the major pathway. However, the involvement of nucleotide excision repair and the overlapping role of repair enzymes does not exclude the role of XP proteins in the repair of DNA damage by styrene or its metabolites (Shimizu et al., 2003; Dusinska et al., 2006).

Abbreviations: SO, Styrene-7,8-oxide; BER, Base-excision repair; PBL, Peripheral blood lymphocytes; Da, Daltons; γ-irradiation, Gamma irradiation; EndoIII sites, SSBs endonuclease III sites; MN, Micronuclei; XRCC1, X-ray repair cross-complementing protein 1; hOGG1, 8-hydroxyguanine DNA glycosylase; XPC, Xeroderma pigmentosum, complementation group C; SSB, Single-strand breaks; bp, Base pair; B2M, Beta-2-Microglobulin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PPIA, Peptidyl-prolyl isomerase A (cyclophilin A); qPCR, Quantitative PCR; SD, Standard deviation.

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Several studies have indicated that individual susceptibility factors, including DNA repair capacity, metabolism and variants in the genes involved may modulate the genotoxicity of xenobiotics (Norppa, 2003, 2004; Vodicka et al., 2004a,c, 2006a). Interestingly, decreased DNA damage, measured as single-strand breaks (SSBs), has been observed with increased styrene exposure in contrast to the BER rates including removal of oxidative-damage, which has been shown to increase in a concentration-dependent manner (Vodicka et al., 2004c).

In order to further elucidate the DNA damage and repair caused by styrene exposure, we have chosen a new population occupationally exposed to styrene at workplace and matched unexposed controls. In this population, we measured SSBs as a marker of DNA damage, frequency of micronuclei (MN) as an indicator of chromosomal damage and studied DNA repair capacity and, to further understand the phenomenon we also measured mRNA level of three repair genes, *XRCC1*, *hOGG1* and *XPC*. Genetic variants in these genes have previously been shown to modulate BER rates in humans (Vodicka et al., 2007).

Materials and methods

Subjects

The styrene-exposed group consisted of 71 workers employed in hand lamination; the mean length of occupational exposure was 5.2 ± 4.0 years (mean ± SD). Fifty-one workers employed as mechanics in a local car plant represented the control group. Thirteen individuals out of 122 (i.e. 10.7%) were also included in the previous sampling (Vodicka et al., 2004c). The set of analyses undertaken was not feasible or successful for all investigated individuals, thus the actual number of observations is shown for each particular parameter in the respective Table and/or Figure. The differences in the styrene exposure were reflected by the stratification of the studied group into three sub-groups according to the level of styrene concentration at workplace. In the control group, the styrene concentration was below the limit of detection. The exposed group was arbitrarily divided into those with low styrene exposure (below 50 mg/m^3) and with the high styrene exposure (above 50 mg/m^3; Table 1). The design of the study was approved by the local ethical committee of Public Health Institute in Usti nad Orlici, Czech Republic. The sampling of biological material was carried out according to the Helsinki declaration.

Styrene exposure at workplace and in blood

The concentration of airborne styrene at workplace was determined by personal dosimeters at the day of sampling (Vodicka et al., 1995). Styrene in blood was determined as previously described (Vodicka et al., 1995, 2001b).

SSBs in DNA

The levels of DNA damage were measured in peripheral blood lymphocytes (PBL) of both the exposed and the control individuals by means of the alkaline version of the comet assay. Using this assay alkali-labile sites may represent alkali-labile DNA adducts, oxidized bases, abasic sites, true DNA breaks as well as transient gaps appearing in the DNA during DNA repair (Vodicka et al., 2006b). The blood samples were kept on ice until processed. PBL were separated using Ficol gradient from the whole blood, rewashed with PBS, re-suspended in low melting point agarose and layered on microscope slides, followed by lysis for 1 h at 4 °C (lysis solution: 2.5 M NaCl, 100 mM EDTA and 10 mM Tris, with 1% Triton X-100, pH 10). In the next step, all slides were treated with alkaline buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 40 min. The electrophoresis was carried out at 25 V, 300 mA for 30 min at 4 °C. All slides were then washed twice with neutralizing buffer (0.4 M Tris, pH 7.5).

To determine abasic sites and oxopyrimidines more specifically, we measured on parallel slides endonuclease III-sensitive sites by incubating lysed nucleoids with endonuclease III enzyme for 45 min at 37 °C. After a 40 min unwinding period, electrophoresis was carried out as above.

For the scoring, slides were stained with ethidium bromide (0.01 ng/μl, 20 μl per agar) and evaluated by an image-analysis system using the comet module of Lucia G image software (Laboratory Imaging, Czech Republic). Fifty randomly selected nuclei per slide were analyzed and the tail DNA percentage was used for calculating amount of DNA breaks according to the calibration of the method with X-ray irradiation and expressed as SSBs/10^9 Da (Collins et al., 1996, 2001; Vodicka et al., 2001a).

DNA repair rates

DNA repair capacity for removal of γ-irradiation-induced SSBs (i.e. BER rates) has been described in details elsewhere. Briefly, PBL,

Table 1
Characteristics of the study population and characterization of styrene exposure. Exposure biomarkers are reported as mean values ± SD, range and median.

		All subjects	Controls	Low exposed	High exposed	p-value[a]
Age	N	122	51	28	43	ns
	Mean ± SD	39 ± 12	40 ± 12	41 ± 12	37 ± 11	
	Range	21–64	26–62	26–64	21–60	
	Median	36.5	37	37	34	
Sex	N males	95	41	28	26	<0.001
	N females	27	10	0	17	
Smoking status	N smokers	72	35	14	23	ns
	N non-smokers	50	16	14	20	
Exposure (Styrene in air; mg/m^3)	N	122	51	28	43	<0.001
	Mean ± SD	50.3 ± 70.3	0.0 ± 0.0	4.9 ± 5.8	139.4 ± 40.2	
	Range	0–238.0	0.0–0.0	0.0–18.0	61.0–238.0	
	Median	0.0	0.0	0.0	152.0	
Years of exposure	N	122	51	28	43	<0.001
	Mean ± SD	3.2 ± 4.0	0.5 ± 1.9	6.9 ± 4.0	4.0 ± 3.6	
	Range	0.0–14.0	0.0–12.0	1.0–13.0	0.5–14.0	
	Median	1.5	0.0	5.3	2.0	
Styrene in blood (mg/l)	N	84	15	27	42	<0.001
	Mean ± SD	1.03 ± 0.98	0.27 ± 0.25	0.41 ± 0.45	1.71 ± 0.94	
	Range	0.00[b]–3.94	0.00[b]–0.76	0.00[b]–2.29	0.33–3.94	
	Median	0.60	0.26	0.31	1.53	

N — number of individuals, SD — standard deviation, ns — not significant.
[a] Level of significance (K–W test).
[b] Value 0 for styrene concentration in blood represents the values below the detection limit, i.e. 0.1 mg/l.

embedded in agarose on microscope slides were irradiated on ice by 5 Gy of gamma rays (0.42 Gy/min). One of the two parallel slides was immediately processed for the comet assay (see above), while the other was first incubated in culture medium (RPMI + 10% foetal calf serum) at 37 °C for 45 min to allow the repair of DNA breaks induced by irradiation. The results (i.e. the amount of repaired SSBs) are calculated as the difference between the initial levels of SSBs, measured immediately after irradiation, and the level of SSBs, detected after 45 min of incubation. The repaired DNA damage is expressed as SSBs/10^9 Da (Vodicka et al., 2004a,b, 2007).

The oxidative DNA repair was analyzed as the capacity of extracts from PBL to repair 8-oxoguanines (Collins et al., 2001). Briefly: aliquots from lymphocyte extracts (from about 10^7 PBL) were pipetted on microscopic slides with HeLa cells mounted in agarose for comet assay. HeLa cells were pretreated with photosensitizer Ro 19-8022 (Hoffmann-La Roche, Basel, Switzerland) and irradiated with fluorescent lamp to induce 8-oxoguanines in the DNA. HeLa cells with induced oxidative DNA damage were incubated with PBL extracts resulting in introduction of SSB at the place of 8-oxoguanines, as described earlier (Vodicka et al., 2004c). Results represent an amount of SSBs created by removal of 8-oxoguanines by specific 8-oxoguanine glycosylase (OGG1) and are expressed as SSBs/10^9 Da (Vodicka et al., 2004c). DNA repair rates for BER and oxidative repair were analyzed in a subset of 97 individuals, due to technical limitations.

Frequency of micronuclei

The technique for the determination of micronuclei (MN) in PBL has been described previously (Migliore et al., 1999). Two thousand binucleated cells for an individual were scored from coded slides for the presence of MN and the results expressed as the frequency of micronucleated binucleated cells (Vodicka et al., 2004c).

Expression analyses

RNA isolation. PBLs were isolated from 10 ml of venous blood and total RNA was immediately isolated using TRIzol according to the procedure supplied by the producer (Invitrogen, Paisley UK). RNA quantity and quality was assessed by UV–VIS spectrophotometry on Carry 300 (Varian Palo Alto, CA www.varianinc.com/cgi-bin/nav) and horizontal agarose gel electrophoresis. cDNA was synthesized using 0.5 or 1 microgram of total RNA by help of RevertAid™ First strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania) with random hexamer primers. As a negative control, we used the same sample, but reverse transcriptase was omitted from the mixture. Quality of cDNA was confirmed by PCR amplification of fragment from control gene ubiquitin C, using primers as reported elsewhere (Soucek et al., 2005). The resulting PCR product indicated whether cDNA derived from mRNA (190 bp) was contaminated by genomic DNA (1009 bp).

qPCR. Expressions of target genes XRCC1, hOGG1 and XPC and reference genes B2M, GAPDH and PPIA were determined by real time PCR in 7500 Real Time PCR System using TaqMan Universal master mix (catalogue no 4364341) and TaqMan Gene Expression Assays 4331182 (Hs00959834_m1 /*XRCC1*/, Hs00213454_m1 /*hOGG1*/, Hs00190295_m1 /*XPC*/), Human *PPIA* Taqman Pre-developed Assay (4333763), TaqMan Endogenous control Human *Beta-2-Microglobulin* (4333766), and TaqMan Endogenous control Human *GAPDH* (4333764). All qPCR reagents were provided by Applied Biosystems (Foster City, CA, USA). As standards for absolute quantification of gene expression, bacterial plasmids pDONR221 containing coding sequences of *XRCC1, hOGG1* and *XPC, B2M, PPIA* and *GAPDH* were used. Constructs were prepared by the Gateway™ cloning technology (Invitrogen, Paisley UK). The presence of the gene fragments in all constructs was confirmed by sequencing (ABI PRISM 310 Genetic Analyzer, Applied Biosystems). cDNA from the samples was diluted 10-times, the expression level was determined as number of copies per μg of total RNA, as previously described (Soucek et al., 2005). The real time PCR was carried out in a final volume of 20 μl containing 5 μl of diluted sample. Cycling program was set at initial hold at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. Results were analyzed using the integrated 7500 System SDS Software version 1.3.1. The expressions of *XRCC1*, *hOGG1* and *XPC* were normalized to the normalization factor calculated from mRNA expression levels of reference genes *B2M*, *PPIA* and *GAPDH* using algorithm GENORM (http://medgen.ugent.be/~jvdesomp/genorm/, wherein the cut off point for the gene expression stability measure M was 0.15.

Primers and cloning specifications are available in the supplementary table 1.

Statistical analyses

Due to asymmetric distribution of the variables of interest, we employed the Kruskal–Wallis (K–W) nonparametric statistical test at bivariate level of analysis. Where appropriate, the distributions of native values were transformed (logarithmic transformation or square root transformation) and parametric tests were applied to test the associations between the studied end-points and explanatory variables both at bivariate and multivariate levels. At this stage of analyses, we used the Pearson correlation analysis, Student T-test and analysis of variance. The simultaneous effects of explanatory variables on the studied end-points were analyzed by means of linear multivariable regression. As independent predictors, we used the variables describing sex, age, smoking, concentration of styrene in blood, duration of exposure in years and mRNA expression levels of studied genes. The adjusted R^2 values and levels of statistical significances are reported for each model. All statistical analyses were carried out using SPSS v 16.0 (SPSS Inc., Chicago, IL, USA).

Results

DNA damage: marker of genotoxic effect

Our results showed higher level of SSBs in DNA from control individuals (1.20 ± 0.70 SSB/10^9 Da) than from the individuals exposed to both low (0.77 ± 0.39 SSB/10^9 Da) and high (0.51 ± 0.41 SSB/10^9 Da) styrene concentrations ($p<0.001$; K–W test, Fig. 1A). In the entire investigated population, a significantly negative correlation was observed between SSBs and styrene concentration at workplace ($R=-0.38$, $p<0.001$), in blood ($R=-0.24$, $p=0.048$) as well as the years of exposure ($R=-0.23$, $p=0.021$). No associations were found when the group was stratified according to exposure. On the other hand, no difference was found in SSB endonuclease III sites between the high and low exposed and the control individuals (Table 2). The duration of exposure expressed in years had no effect on SSB endonuclease III sites. The levels of both SSBs and SSB endonuclease III sites were not affected by smoking, as analyzed in the whole group.

Frequency of micronuclei

Frequencies of MN were not significantly modulated by styrene exposure (6.8 ± 4.5‰ in control persons, 7.2 ± 2.1‰ in workers exposed to low styrene concentration and 7.3 ± 4.6‰ in those exposed to high styrene concentrations, respectively; Table 2). Further, we did not record any associations between MN and SSBs levels, DNA repair rates or mRNA expression levels of the studied genes. The only parameters having effect on MN frequency were gender and age ($R=0.41$, $p<0.001$, $R=0.50$, $p<0.001$, respectively, Pearson).

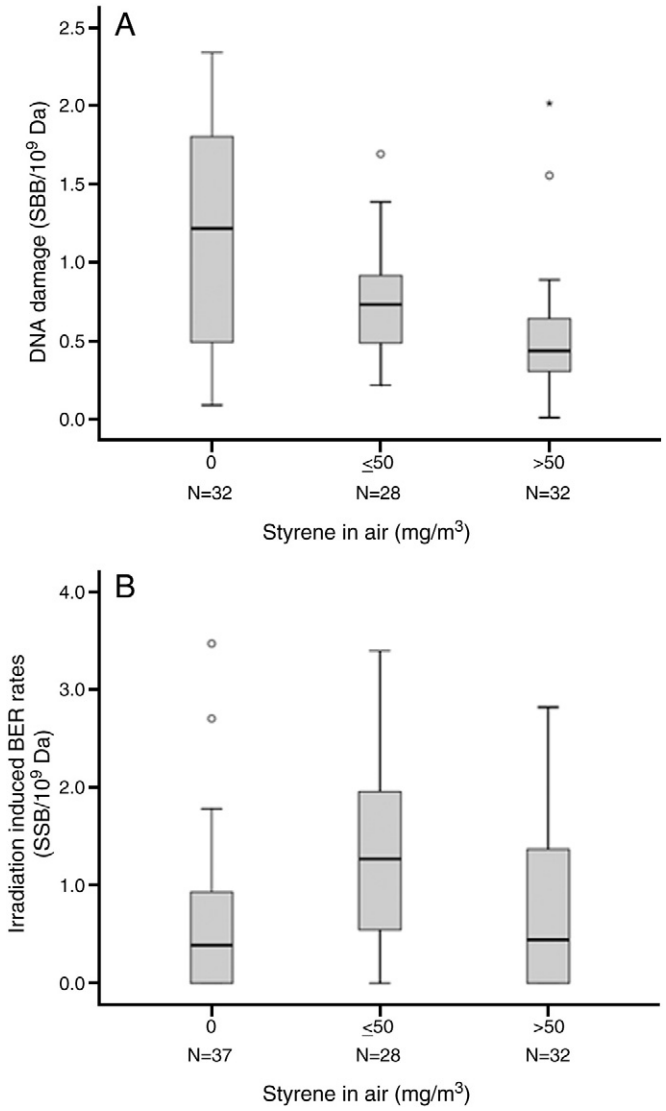

Fig. 1. SSB level and γ-irradiation-specific DNA repair capacity both expressed as SSB/10^9 Da with respect to exposure. A) SSB level is higher in the control group (1.20 ± 0.70 SSB/10^9 Da) and lower in the low and high exposed groups (0.77 ± 0.39 SSB/10^9 Da, 0.51 ± 0.41 SSB/10^9 Da respectively, $p<0.001$, K-W test); B) the γ-irradiation-specific DNA repair rates are higher in the low exposed group (1.34 ± 1.00 SSB/10^9 Da) and lower in the control and high exposed groups (0.65 ± 0.82 SSB/10^9 Da and 0.72 ± 0.81 SSB/10^9 Da respectively, $p=0.005$, K-W test). Bars represent mean ± standard deviation.

Functional tests on DNA repair

The γ-irradiation-specific DNA repair rates were the highest among individuals with low styrene exposure (1.34 ± 1.00 SSB/10^9 Da), followed by those with high styrene exposure (0.72 ± 0.81 SSB/10^9 Da) and the controls (0.65 ± 0.82 SSB/10^9 Da); the differences between the groups were statistically significant ($p=0.005$; K–W test; Fig. 1B). No significant difference was observed in the capacity to repair oxidative DNA damage between the exposed and the control individuals ($p=0.094$, K–W test, Table 2).

The γ-irradiation-specific DNA repair rates were elevated in relation to the duration of exposure ($R=0.34$, $p=0.001$). Sex, age, smoking, concentration of styrene in blood and at workplace did not modulate either γ-irradiation-specific DNA repair rates or the capacity to repair oxidative DNA damage.

We did not find any association between γ-irradiation-specific DNA repair rates and SSBs either in the whole group or after stratification according to the levels of exposure. Similarly, no association was revealed between γ-irradiation-specific DNA repair rates and SSB endonuclease III sites in the whole group or after stratification.

Expression levels of XRCC1, hOGG1 and XPC

In bivariate statistical analyses, the expression levels of *XRCC1*, *hOGG1* and *XPC* were significantly lower in the exposed groups as compared to the control group ($p<0.001$ for each of the genes, K–W test, Table 2). Expression levels of *XRCC1*, *hOGG1* and *XPC*, based on the analysis of the whole study population, negatively correlated with styrene concentration in blood ($R=-0.40$, $p<0.001$ for *XRCC1*, $R=-0.66$, $p<0.001$ for *hOGG1*, and $R=-0.36$, $p<0.001$ for *XPC*, Pearson).

We observed that both SSBs and SSBs endonuclease III sites increased with the increasing mRNA expression levels of *XRCC1*, *hOGG1* and *XPC* (Table 3). Only expression levels of *XPC* negatively correlated with γ-irradiation-specific DNA repair capacity ($R=-0.21$, $p=0.047$; Table 3).

Expression level of *XPC* moderately increased with smoking ($R=0.27$, $p=0.041$; Pearson). Our data did not show any correlation between age and the expression of any gene studied.

Outcomes from multivariate statistical evaluations

DNA damage and DNA repair rates (irradiation–as well as oxidative damage–induced) were analyzed in multivariate regression models. Out of all dependent variables tested, which included gender, age, smoking, concentration of styrene in blood, duration of exposure and mRNA expression levels of the investigated genes, the SSB levels were significantly affected by gender and *hOGG1* mRNA expression (Table 4). SSB endonuclease III sites were affected by *XRCC1* expression along with the duration of styrene exposure (see Table 4).

The γ-irradiation-specific DNA repair rates were significantly affected by duration of exposure (Table 4), whereas the oxidative repair rates were not modified by any of the investigated factors.

Discussion

In this study, we observed exposure-related decrease in SSB levels and mRNA expression levels in *XRCC1*, *hOGG1* and *XPC*, while the DNA repair capacity was the highest in the low exposed group, followed by the high exposed group and the lowest in the controls. Our earlier study also showed decreased DNA damage with increasing concentrations of styrene in air, whereas the capacity to repair irradiation-induced specific DNA damage (reflecting mainly BER activity) was significantly higher in exposed subjects. We postulated the possible induction of repair mechanisms due to styrene exposure (Vodicka et al., 2004c). The present study has shown that induction of BER most probably does not proceed via transcriptional activation. A recent study on 34 styrene-exposed workers and 29 controls reported an exposure-related increase in SSBs, accompanied with a decrease in DNA repair capacity (Fracasso et al., 2009). These findings are in line with earlier studies, describing an increase of genotoxicity markers with styrene exposure reviewed in (Vodicka et al., 2006a), but in contrast with earlier findings (Vodicka et al., 2004c) and with the present data. We did not observe any tendency of suppression of DNA repair as reported by (Fracasso et al., 2009) for workers exposed to more than 200 mg/m^3 styrene on average and accompanied by an accumulation of SSBs. A putative threshold in DNA repair rates was suggested in our earlier study (Vodicka et al., 2003), where an increase in BER rates was observed in individuals exposed to styrene up to 100 mg/m^3, followed by a decrease at higher styrene concentrations. Both our present data on DNA repair capacity as well as those by (Fracasso et al., 2009) seem to be in line with the above assumption. Although in the present study we did not confirm previous

Table 2
Parameters of genotoxicity, DNA repair rates and mRNA expression levels in DNA repair genes. Values are expressed as mean ± SD, range and median.

		All subjects	Controls	Low exposed	High exposed	p-value[a]
SSBs in DNA (SSB/10^9Da)	N	97	37	28	32	<0.001
	Mean ± SD	0.85 ± 0.61	1.20 ± 0.70	0.77 ± 0.39	0.51 ± 0.41	
	Range	0.01–2.30	0.10–2.30	0.20–1.70	0.00–2.00	
	Median	0.65	1.22	0.73	0.44	
Endo*III* sites (SSB/10^9Da)	N	97	37	28	32	
	Mean ± SD	0.46 ± 0.44	0.51 ± 0.55	0.52 ± 0.43	0.33 ± 0.27	ns
	Range	0.00–2.03	0.00–2.03	0.00–1.59	0.00–1.02	
	Median	0.37	0.28	0.49	0.29	
γ-irradiation DNA repair rates (SSB/10^9Da)	N	97	37	28	32	0.005
	Mean ± SD	0.87 ± 0.91	0.65 ± 0.82	1.34 ± 1.00	0.72 ± 0.81	
	Range	0.00–3.00	0.00–3.00	0.01–3.00	0.00–3.00	
	Median	0.61	0.39	1.27	0.44	
Repair rates oxidative DNA damage	N	97	36	27	34	
(SSB/10^9Da)	Mean ± SD	0.43 ± 0.43	0.45 ± 0.42	0.55 ± 0.51	0.32 ± 0.36	ns
	Range	0.00–1.80	0.00–1.49	0.00–1.08	0.00–1.60	
	Median	0.29	0.27	0.32	0.17	
Binucleated cells with MN (‰)	N	112	50	27	35	ns
	Mean ± SD	7.1 ± 4.1	6.8 ± 4.5	7.2 ± 2.1	7.3 ± 4.6	
	Range	2.0 ± 27.5	2.0 ± 25.5	4.0 ± 12.0	2.0 ± 27.5	
	Median	6.5	6.0	6.5	7.0	
Expression levels of XPC	N	118	49	28	41	
(number of copies per 1 μg of total RNA)	Mean ± SD	$2.81\times10^6 \pm 1.30\times10^6$	$3.72\times10^6 \pm 1.48\times10^6$	$2.42\times10^6 \pm 0.70\times10^6$	$2.00\times10^6 \pm 0.46\times10^6$	<0.001
	Range	1.02×10^6–8.55×10^6	1.19×10^6–8.55×10^6	1.10×10^6–4.09×10^6	1.02×10^6–2.85×10^6	
	Median	2.38×10^6	3.69×10^6	2.33×10^6	1.98×10^6	
Expression levels of XRCC1	N	122	51	28	43	<0.001
(number of copies per 1 μg of total RNA)	Mean ± SD	$3.00\times10^6 \pm 1.79\times10^6$	$4.37\times10^6 \pm 1.94\times10^6$	$2.28\times10^6 \pm 0.65\times10^6$	$1.85\times10^6 \pm 0.71\times10^6$	
	Range	0.88×10^6–9.50×10^6	1.42×10^6–9.50×10^6	1.51×10^6–4.62×10^6	0.88×10^6–4.97×10^6	
	Median	2.19×10^6	3.93×10^6	2.09×10^6	1.79×10^6	
Expression levels of hOGG1	N	122	51	28	43	<0.001
(number of copies per 1 μg of total RNA)	Mean ± SD	$6.58\times10^6 \pm 3.07\times10^6$	$9.38\times10^6 \pm 2.02\times10^6$	$6.42\times10^6 \pm 1.31\times10^6$	$3.35\times10^6 \pm 0.95\times10^6$	
	Range	2.39×10^6–13.22×10^6	4.63×10^6–13.22×10^6	3.05×10^6–8.47×10^6	2.39×10^6–6.87×10^6	
	Median	6.43×10^6	9.80×10^6	6.64×10^6	3.08×10^6	

N — number of individuals; SD — standard deviation; ns — not significant.
[a] Level of significance (K–W test).

findings on an increased DNA repair response to styrene-induced genotoxicity (Vodicka et al., 2004c), a positive significant correlation between γ-irradiation-specific DNA repair rates and duration of exposure may indicate an increased BER capacity with a cumulative exposure. Surprisingly, the main increase in BER capacity was observed in a group with low styrene exposure. A further stratification for a cumulative exposure (by multiplying styrene concentration in ambient air and years of exposure) did not reveal any significant differences in γ-irradiation-specific DNA repair rates (data not shown). However, only limited information is available on the cascade of exposure to xenobiotics, DNA damage and DNA repair functional response both in experimental animals and humans (Vodicka et al., 2004a,c, 2006b) and these aspects remain to be elucidated.

In the present study the MN frequencies were not affected by styrene exposure. Although some studies point out to the increased MN frequencies in relation to styrene exposure (Vodicka et al., 2004c; Migliore et al., 2006), the last reviews on styrene assessed the association between cytogenetic markers (including MN) and styrene exposure as inconclusive (Vodicka et al., 2006a; Rueff et al., 2009). However, MN frequencies were increased in women and in older subjects, as convincingly described earlier (Fenech, 1998).

We did not discover any associations between γ-irradiation-specific DNA repair rates and mRNA expression levels of three important DNA repair genes (*XRCC1*, *hOGG1* and *XPC*).

Our results showed that the expression levels of *XRCC1*, *hOGG1* and *XPC* were significantly lower in the exposed individuals than in the controls. There is no simple mechanistic explanation for an exposure-related decrease of mRNA expression levels in the studied DNA repair genes in humans at present. However, some *in vitro* studies indicated that oxidative/genotoxic stress-generating agents do not necessarily modify the mRNA expression levels of DNA repair genes. For instance, treatment of HeLa cells with oxidative stress-generating agents did not

Table 3
Correlation between parameters of DNA damage and mRNA expression levels.

mRNA gene expression levels[a]	Parameters of DNA damage				γ-irradiation-specific DNA repair capacity[b]	
	SSB[b]		EndoIII sites[b]			
	R	p-value	R	p-value	R	p-value[c]
hOGG1	0.34	<0.001	0.38	0.002		ns
N		95		74		95
XPC	0.35	<0.001	0.31	0.007	−0.21	0.047
N		93		73		93
XRCC1	0.30	<0.001	0.38	0.001		ns
N		95		74		95

ns — not significant, N — number of individuals.
[a] Log transformed data.

Table 4
Outcomes from the multivariate regression statistics.

Studied end-points	Explanatory variables[a]	B	Adjusted R^2	p-value	N
SSB[c]	Sex (0 for men, 1 for women)	−0.31	0.33	0.001	92
	hOGG1 expression[b]	0.35		0.017	
Endo III sites[c]	*XRCC1* expression[b]	0.61	0.20	0.001	72
	Duration of exposure in years	0.02		0.040	
BER rates[c]	Duration of exposure in years	0.04	0.09	0.003	92

N — number of individuals.
[a] Explanatory variables included sex, age, smoking, concentration of styrene in blood, duration of exposure in years, and mRNA expression levels of studied genes.
[c] Square root transformed data.
[b] Log transformed values.

alter the mRNA expression level of *hOGG1* (Dhenaut et al., 2000). Recently, a potassium bromate-induced increase in the activity of OGG1 in cells was not accompanied by an increase in *OGG1* gene expression as assessed by qPCR, suggesting a role of protein stabilization or elevated OGG1 catalytic activity (Mirbahai et al, 2009.). A decrease of mRNA expression of *XRCC1* (to 20–40%) was recorded following the treatment with 4-nitroquinoline-1-oxide, and also UV and ionizing radiation failed to show induction of the mRNA for *XRCC1* (Yoo et al., 1992).

In the study by (Manini et al., 2009) *hOGG1* expression levels increased with the exposure to styrene. The difference in expression of *hOGG1* in comparison to the present study may mainly be ascribed to the normalization of *hOGG1* gene expression to only one reference gene *GAPDH*. This particular reference gene was used in a previous study (Diodovich et al., 2004) and proven not to be influenced by styrene treatment. Additionally, there were differences in number of studied individuals between the two studies.

For an effective elimination of genotoxic burden, the cells must not necessarily increase expression of DNA repair genes at the mRNA level. DNA repair is a complex process, modified by DNA damage sensing and signaling (Harper and Elledge, 2007), and is regulated at various levels, e.g. transcriptional regulations, post-translational modifications. Furthermore, modifications of BER proteins, including phosphorylation, acetylation, ubiquitination, etc., may also dramatically affect organelle targeting and repair activity (Fan and Wilson, 2005; Hazra et al., 2007).

We observed that both SSBs and SSBs endonuclease III sites increased with the increasing mRNA expression levels of *XRCC1*, *hOGG1* and *XPC*, but not with γ-irradiation-specific DNA repair rates. It is therefore difficult to ascribe arising SSBs to the intermediates of DNA repair (as SSBs reflect levels of DNA strand breaks, apurinic/apyrimidinic sites as well as intermediates in the repair process (Collins, 2009).

Among the analyzed confounders the only significant correlation was detected between smoking and *XPC* expression. This finding is in accordance with a recent study, where *XPC* mRNA levels induced by ionizing radiation were substantially affected by smoking (Wiebalk et al., 2007).

This present study has an advantage of investigating the end-points of styrene genotoxicity in relation with DNA repair capacity and simultaneous measurement of mRNA expression levels in three relevant DNA repair genes. The selection of DNA repair genes of interest was based on our previous studies (Vodicka et al., 2004b, 2007), reporting the links between genotypes in *XRCC1*, *hOGG1*, and, partially, *XPC* and γ-irradiation-specific DNA repair rates.

Obtained data may contribute to our understanding of styrene-induced genotoxicity and the response of DNA repair system, assayed for by the determination of mRNA expression levels of XRCC1, hOGG1 and XPC genes, and BER rates. Understanding the molecular responses to genotoxic stress requires further highly-targeted study, aimed mainly at discerning the role of DNA damage sensing, DNA repair pathways (functional tests) and the regulation, function and modification of the relevant proteins/enzymes at the protein level in the cells following the exposure to xenobiotics.

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Manuscript IV

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DNA damage, DNA repair rates and mRNA expression levels of cell cycle genes (*TP53*, *p21^{CDKN1A}*, *BCL2* and *BAX*) with respect to occupational exposure to styrene

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We studied the relationship between DNA damage, DNA repair rates and messenger RNA (mRNA) expression levels of cell cycle genes *TP53*, *p21^{CDKN1A}*, *BCL2* and *BAX* in a group of 71 styrene-exposed workers and 51 control individuals. The exposure was assessed by measuring the concentration of styrene at workplace and in blood. Parameters of DNA damage [measured as single-strand breaks (SSBs) and endonuclease III-sensitive sites], γ-irradiation-specific DNA repair rates and mRNA levels of studied genes were analyzed in peripheral blood lymphocytes. The workers were divided into low (<50 mg/m^3) and high (>50 mg/m^3) styrene exposure groups. We found negative correlations between mRNA expression of *TP53*, *BCL2*, *BAX* and styrene exposure ($P < 0.001$ for all parameters). In contrast, *p21^{CDKN1A}* mRNA expression significantly increased with increasing styrene exposure ($P = 0.001$). SSBs and endonuclease III-sensitive sites increased with increasing mRNA levels of *TP53* ($P < 0.001$ for both) and *BCL2* ($P = 0.038$, $P = 0.002$, respectively), whereas the same parameters decreased with increasing mRNA levels of *p21^{CDKN1A}* ($P < 0.001$, $P = 0.007$, respectively). γ-Irradiation-specific DNA repair rates increased with *p21^{CDKN1A}* mRNA levels up to the low exposure level ($P = 0.044$). Our study suggests a possible relationship between styrene exposure, DNA damage and transcript levels of key cell cycle genes.

Introduction

Styrene, a monomer widely used in chemical industries for the production of various plastic and polyester resins, has comprehensively been reviewed for its genotoxicity and potential carcinogenicity (1–3).

Several studies have indicated that individual susceptibility factors, including DNA repair capacity, metabolic capacity and variants in the genes involved in these processes, may modulate the genotoxicity of xenobiotics (4–7). However, only limited data are available on the mechanisms of DNA damage sensing/response in humans exposed to xenobiotics (8). Very recently, decreased DNA damage (measured as single-strand breaks; SSBs) has been observed with increased styrene exposure and was not accompanied by an increase in DNA repair rates, reflecting mainly base excision repair (BER). Interestingly, expression levels in *XRCC1*, *XPC* and *hOGG1* genes decreased with increasing styrene exposure in hand lamination workers (9).

The DNA damage response pathway is a multicomponent signal transduction network that consists of a multitude of proteins. TP53 and p21^{CDKN1A} have a role in DNA damage sensing and signaling pathways, and their activity results in growth arrest and apoptosis in response to a range of both environmental and intracellular genotoxic stresses (10). p21^{CDKN1A} is a cyclin-dependent kinase inhibitor, its expression is controlled by TP53, and it also exerts a regulatory role in BER (11,12).

BCL2 family of mammalian genes acts as anti- or pro-apoptotic regulators, involved in a wide variety of cellular activities. *BCL2* encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes. BAX protein forms a heterodimer with BCL2 and functions as an apoptotic activator (13).

Only two *in vitro* studies have investigated expression levels of specific cell cycle genes with respect to genotoxic stress induced by styrene and its main intermediate, styrene-7,8-oxide (14,15). When studying the effect of styrene-7,8-oxide on messenger RNA (mRNA) expression levels of *TP53*, *p21^{CDKN1A}*, *BAX* and *BCL2* in cultured human peripheral blood lymphocytes (PBLs), no clear up- or down-regulation in any of the genes was recorded after treatment (14). In contrast, *in vitro* styrene administration to the cord blood cells resulted in a clear overexpression of BCL2, whereas BAX was down-regulated 6 h after the treatment and p*53* protein expression was not affected (15).

Based on the previous studies and due to the lack of information on the mRNA expression of the above genes in individuals occupationally exposed to styrene, we have studied gene expressions of the DNA damage response genes *TP53*, *p21^{CDKN1A}*, *BCL2* and *BAX* in PBL. Further, mRNA expression levels were evaluated in context with DNA damage and γ-irradation-specific DNA repair to reflect mechanism of interactions following the exposure to xenobiotics in humans.

Materials and methods

Subjects

The styrene-exposed group consisted of 71 workers employed as hand lamination workers for 5.2 ± 4.0 years (mean ± SD). Fifty-one workers employed as mechanics in a local car plant represented the control group. The set of analyses undertaken was not feasible or successful for all investigated individuals, thus the actual number of observations is shown for each particular parameter in the respective Table I and III and/or Figure 1 and 2. The differences in the styrene exposure were reflected by the stratification of the studied group into three subgroups according to the level of styrene concentration at workplace. The exposed group was arbitrarily divided into those with low styrene exposure (<50 mg/m^3) and with the high styrene exposure (>50 mg/m^3; Table I). The design of the study was approved by the Ethics Committee of the Public Health Institute in Usti nad Orlici, Czech Republic. The sampling of biological material was carried out according to the Helsinki declaration.

Styrene exposure at workplace and in blood

The concentration of airborne styrene at workplace was determined by personal dosimeters at the day of sampling (16). Styrene in blood was determined as described previously (16,17).

DNA damage and DNA repair rates

DNA damage was measured as SSBs and more specifically as endonuclease III-sensitive sites (EndoIII sites). SSBs in PBL of both the exposed and the control individuals by means of the alkaline version of the comet assay. Using this assay, alkali-labile sites may represent alkali-labile DNA adducts, oxidized bases, abasic sites, true DNA breaks as well as transient gaps appearing in the

Abbreviations: BER, base excision repair; cDNA, complementary DNA; mRNA, messenger RNA; PBL, peripheral blood lymphocyte; PCR, polymerase chain reaction; SSB, single-strand break.

Table I. Characteristics of the study population and styrene exposure

		All subjects	Controls	Low exposed	High exposed	*P*-value [a]
Age	*N*	122	51	28	43	ns
	Mean ± SD	39 ± 12	40 ± 12	41 ± 12	37 ± 11	
	Range	26–64	26–62	26–64	21–60	
	Median	36.5	37	37	34	
Sex	*N* males	95	41	28	26	<0.001
	N females	27	10	0	17	
Smoking status	*N* smokers	72	35	14	23	ns
	N non-smokers	50	16	14	20	
Exposure (styrene in air; mg/m^3)	*N*	122	51	28	43	<0.001
	Mean ± SD	50.3 ± 70.3	0.0 ± 0.0	4.9 ± 5.8	139.4 ± 40.2	
	Range	0–238.0	0.0–0.0	0.0–18.0	61.0–238.0	
	Median	0.0	0.0	0.0	152.0	
Styrene in blood (mg/l)	*N*	84	1.5	2.7	42	<0.001
	Mean ± SD	1.03 ± 0.98	0.27 ± 0.25	0.41 ± 0.45	1.71 ± 0.94	
	Range	0.00[b]–3.94	0.00[b]–0.76	0.00[b]–2.29	0.33–3.94	
	Median	0.60	0.26	0.31	1.53	

Exposure biomarkers are reported as mean values ± SD, range and median. *N*, number of individuals; SD, standard deviation; ns, not significant.
[a]Level of significance (K–W test).
[b]Value 0 for styrene concentration in blood represents the values below the detection limit, i.e. 0.1 mg/l.

DNA during DNA repair (18). The blood samples were kept on ice until processed. PBL were separated using Ficoll gradient from the whole blood, rewashed with phosphate-buffered saline, resuspended in low melting point agarose and layered on microscope slides, followed by lysis for 1 h at 4°C (lysis solution: 2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid and 10 mM Tris, with 1% Triton X-100; pH 10). In the next step, all slides were treated with alkaline buffer (300 mM NaOH, 1 mM ethylenediaminetetraacetic acid; pH 13) for 40 min. The electrophoresis was carried out at 25 V, 300 mA for 30 min at 4°C. All slides were then washed twice with neutralizing buffer (0.4 M Tris, pH 7.5).

To determine abasic sites and oxopyrimidines more specifically, we measured on parallel slides endonuclease III-sensitive sites by incubating lysed nucleoids with endonuclease III enzyme for 45 min at 37°C. After a 40 min unwinding period, electrophoresis was carried out as above.

For the scoring, slides were stained with ethidium bromide (0.01 ng/µl, 20 µl/agar) and evaluated by an image-analysis system using the comet module of Lucia G image software (Laboratory Imaging, Prague, Czech Republic). Fifty randomly selected nuclei per slide were analyzed and the tail DNA percentage was used for calculating amount of DNA breaks according to the calibration of the method with X-ray irradiation and expressed as SSBs/10^9 Da (19–21).

DNA repair capacity for removal of γ-irradiation-induced SSBs (i.e. BER rates) has been described in details elsewhere. Briefly, PBL, embedded in agarose on microscope slides were irradiated on ice by 5 Gy of gamma rays (0.42 Gy/min). One of the two parallel slides was immediately processed for the comet assay (see above), whereas the other was first incubated in culture medium (RPMI + 10% fetal calf serum) at 37°C for 45 min to allow the repair of DNA breaks induced by irradiation. The results (i.e. the amount of repaired SSBs) are calculated as the difference between the initial levels of SSBs, measured immediately after the irradiation, and the level of SSBs, detected after 45 min of incubation. The repaired DNA damage is expressed as SSBs/10^9 Da (6,7,22). DNA repair rates for BER were analyzed in a subset of 97 individuals, due to technical limitations.

Expression analyses

RNA isolation. PBLs were isolated from 10 ml of venous blood and total RNA was immediately isolated using TRIzol according to the procedure supplied by the manufacturer (Invitrogen, Paisley, UK). RNA quantity and quality was assessed by ultraviolet-visible spectrophotometry on Cary 300 (Varian, Palo Alto, CA) and horizontal agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized using 0.5 or 1 µg of total RNA by help of RevertAidTM First strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania) with random hexamer primers. As a negative control, we used the same sample, but reverse transcriptase was omitted from the mixture. Quality of cDNA was confirmed by polymerase chain reaction (PCR) amplification of ubiquitin C fragment using primers as reported elsewhere (23).

Quantitative PCR. Expressions of target genes TP53, p21^{CDKN1A}, BCL2 and BAX and reference genes *B2M*, *GAPDH* and *PPIA* were determined by real time PCR in 7500 Real Time PCR System using TaqMan Universal master mix (catalogue no 4364341) and TaqMan Gene Expression Assays 4331182 [Hs00153349_m1/*TP53*/, Hs00355782_m1/*CDKN1A*/, Hs00153350_m1/*BCL2*/, Hs00180269_m1/BAX/, Human PPIA Taqman Pre-developed Assay (4333763), TaqMan Endogenous control Human Beta-2-Microglobulin (4333766), and TaqMan Endogenous control Human GAPDH (4333764)]. All quantitative PCR reagents were provided by Applied Biosystems (Foster City, CA). As standards for absolute quantification of gene expression, bacterial plasmids pDONR221 containing coding sequences of *BCL2* and *BAX*, *B2M*, *PPIA* and *GAPDH* were used. Constructs were prepared by the Gateway^{TM} cloning technology (Invitrogen). As standards for *p21^{CDKN1A}* and *TP53* genes, the PCR products were used. All primers were designed using free software available at http://frodo.wi.mit.edu/primer3 and are stated in Table II. The presence of the gene fragments in all constructs was confirmed by sequencing (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). cDNA from the samples was diluted 10 times; the expression level was determined as number of copies per microgram of total RNA, as described previously (23). The real time PCR was carried out in a final volume of 20 µl containing 5 µl of diluted cDNA. Cycling program was set at initial hold at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. Results were analyzed using the integrated 7500 System SDS Software version 1.3.1. The expressions of *TP53*, *p21^{CDKN1A}*, *BCL2* and *BAX* were normalized to the normalization factor calculated from mRNA expression levels of reference genes *B2M*, *PPIA* and *GAPDH* using algorithm GENORM (http://medgen.ugent.be/~jvdesomp/genorm/). The cut off point for the gene expression stability measure M was set to 0.15.

Table II. Primers for genes of interest (*TP53*, *p21^{CDKN1A}*, *BCL2* and *BAX*) and reference genes (*PPIA*, *B2M* and *GAPDH*)

Gene name	Primer	Sequence
TP53	Forward	GTGGAAGGAAATTTGCGTGT
	Reverse	TTTGGGTCTTTGAACCCTTG
p21^{CDKN1A}	Forward	GGAAGACCATGTGGACCTGT
	Reverse	TTCCTAAGAGTGCTGGGCAT
BCL2	Forward[a]	**ACCATATG**ATGTGTGTGGAGAGCGTCAA
	Reverse[b]	**AGTCGAC**TTTTCCATCCGTCTGCTCTT
BAX	Forward[a]	**ACCATATG**TTTGCTTCAGGGTTTCATCC
	Reverse[b]	**AGTCGAC**CTCAGCCCATCTTCTTCCAG
PPIA	Forward[a]	**ACCATATG**AGGGTTCCTGCTTTCACAGA
	Reverse[b]	**AGTCGAC**CCCAGTTGCTGCCTACATTT
B2M	Forward[a]	**ACCATATG**GTGCTCGCGCTACTCTCTCT
	Reverse[b]	**AGTCGAC**TCTCTGCTCCCCACCTCTAA
GAPDH	Forward[a]	**ACCATATG**CTCTGCTCCTCCTGTTCGAC
	Reverse[b]	**AGTCGAC**TTCTAGACGGCAGGTCAGGT

Restriction sites highlighted in bold.
[a]Forward primer, restriction site for: NdeI.
[b]Reverse primer, restriction site for: SalI.

Table III. Correlation between markers of exposure and mRNA expression levels (tested in the whole study population)

Markers of exposure		mRNA gene expression levels[a]			
		BAX	*BCL2*	*p21^{CDKN1A}*	*TP53*
Styrene concentration at workplace	Spearman's coefficient	−0.48	−0.65	0.64	−0.75
	P[b]	<0.001	<0.001	<0.001	<0.001
	N	121	122	122	122
Styrene concentration in blood	Spearman's coefficient	−0.50	−0.62	0.40	−0.74
	P[b]	<0.001	<0.001	<0.001	<0.001
	N	83	84	84	84

N, number of individuals.
[a]Logarithmically transformed data.
[b]Level of significance.

Statistical analyses

Due to asymmetric distribution of the variables of interest, we employed the Kruskal–Wallis (K–W) nonparametric statistical test and Spearman correlation at bivariate level of analysis. When appropriate, the distributions of native values were transformed (logarithmic transformation or square root transformation) and parametric tests were applied to test the associations between the studied end points and explanatory variables. At this stage of analyses, we used the Pearson correlation analysis and the analysis of variance. All statistical analyses were carried out using SPSS version 16.0 (SPSS, Chicago, IL).

Results

Effect of styrene exposure on mRNA levels of TP53, p21^{CDKN1A}, BCL2 and BAX

The relationship between markers of exposure (concentrations of styrene in blood and at workplace) and mRNA expression levels of *TP53*, *p21^{CDKN1A}*, *BCL2* and *BAX* was tested. Significant

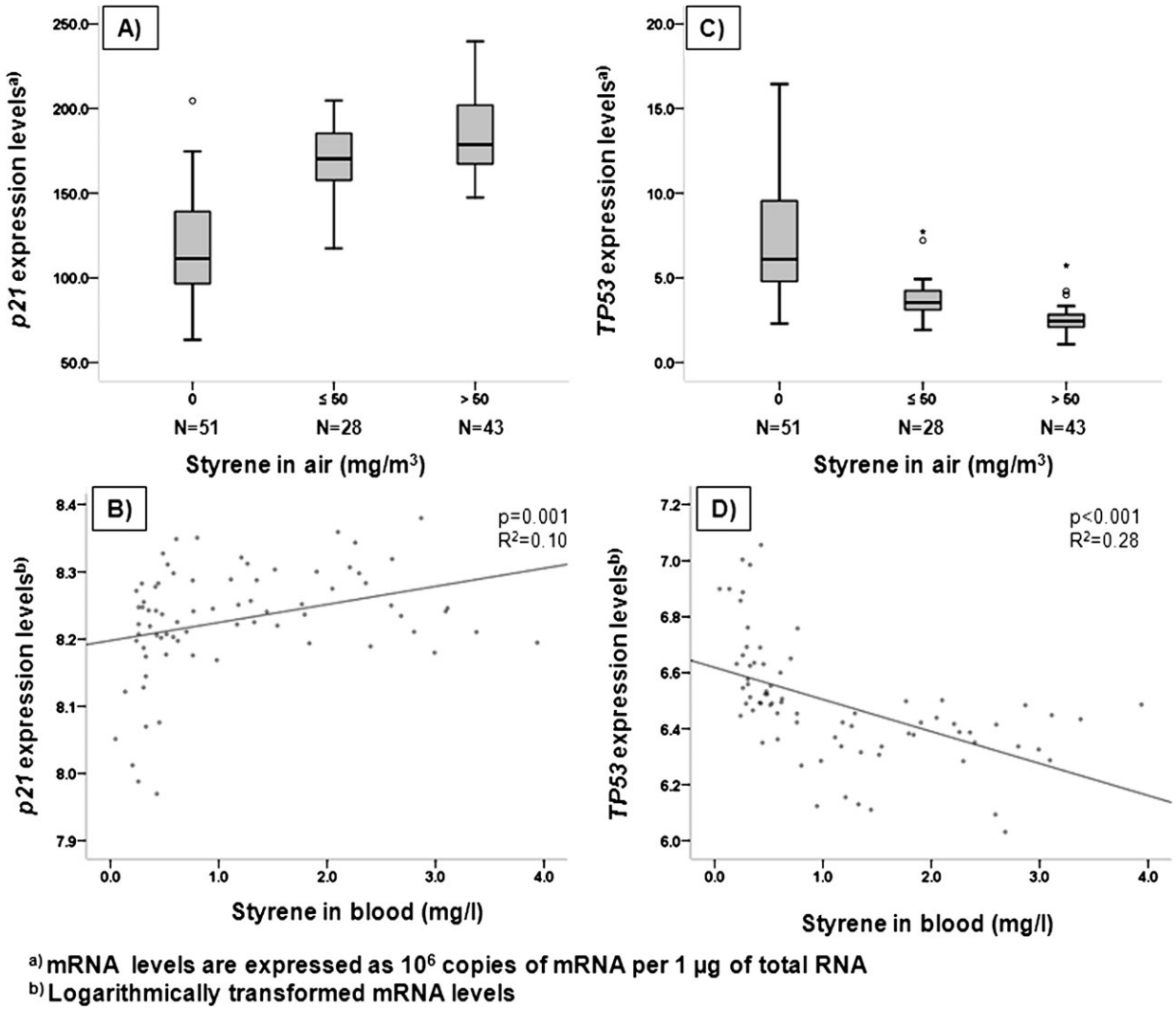

Fig. 1. mRNA levels of *p21^{CDKN1A}* and *TP53* in the study population stratified according to styrene exposure. (**A**) The decrease of mRNA expression levels of *p21^{CDKN1A}* with increasing exposure is shown. The expression decreases from $1.17 \times 10^8 \pm 2.80 \times 10^7$ copies per 1 µg of total RNA in the control group to $1.69 \times 10^8 \pm 2.12 \times 10^7$ copies per 1 µg of total RNA in the low exposed and $1.85 \times 10^8 \pm 2.35 \times 10^7$ copies per 1 µg of total RNA in the high exposed groups ($P < 0.001$, K–W test). Bars represent mean ± standard deviation. (**B**) The correlation between logarithmically transformed mRNA expression levels of *p21^{CDKN1A}* and concentration of styrene in blood (mg/ml) is shown ($P = 0.001$, $R = 0.37$, Spearman, $R^2 = 0.10$). (**C**) The decrease of mRNA expression levels of *TP53* with increasing exposure is shown. The expression decreases from $7.01 \times 10^6 \pm 3.23 \times 10^6$ copies per 1 µg of total RNA in the control group to $3.82 \times 10^6 \pm 1.24 \times 10^6$ copies per 1 µg of total RNA in the low exposed and $2.50 \times 10^6 \pm 0.84 \times 10^6$ copies per 1 µg of total RNA in the high exposed groups ($P < 0.001$, K–W test). Bars represent mean ± standard deviation. (**D**) The correlation between logarithmically transformed mRNA expression levels of *TP53* and concentration of styrene in blood (milligrams per microliter) is shown ($P < 0.001$, $R = -0.713$, Spearman, $R^2 = 0.28$).

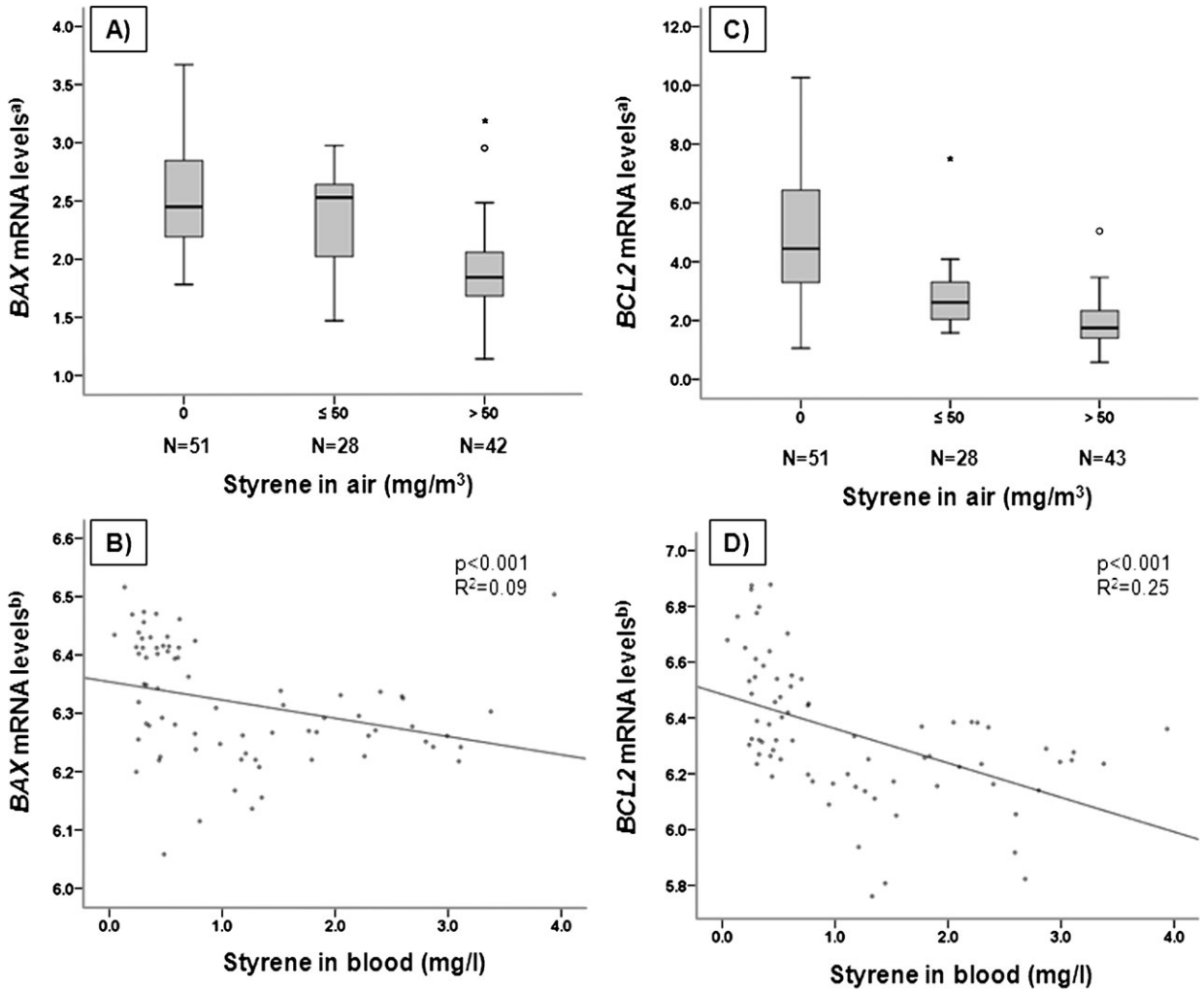

Fig. 2. mRNA levels of *BAX* and *BCL2* in the study population stratified according to styrene exposure. (**A**) The decrease of mRNA expression levels of *BAX* with increasing styrene exposure is shown. The expression decreases from $2.54 \times 10^6 \pm 0.51 \times 10^6$ copies per 1 µg of total RNA in the control group to $2.36 \times 10^6 \pm 0.42 \times 10^6$ copies per 1 µg of total RNA in the low exposed and $1.91 \times 10^6 \pm 0.39 \times 10^6$ copies per 1 µg of total RNA in the high exposed groups ($P < 0.001$, K–W test). Bars represent mean ± standard deviation. (**B**) The correlation between logarithmically transformed mRNA expression levels of *BAX* and concentration of styrene in blood (milligrams per microliter) is shown ($P < 0.001$, $R = -0.457$, Spearman, $R^2 = 0.09$). (**C**) The decrease of mRNA expression levels of *BCL2* with increasing exposure is shown. The expression decreases from $4.84 \times 10^6 \pm 2.26 \times 10^6$ copies per 1 µg of total RNA in the control group to $2.80 \times 10^6 \pm 1.19 \times 10^6$ copies per 1 µg of total RNA in the low exposed and $1.85 \times 10^6 \pm 0.83 \times 10^6$ copies per 1 µg of total RNA in the high exposed groups ($P < 0.001$, K–W test). Bars represent mean ± standard deviation. (**D**) The correlation between logarithmically transformed mRNA expression levels of *BCL2* and concentration of styrene in blood (milligrams per microliter) is shown ($P < 0.001$, $R = -0.619$, Spearman, $R^2 = 0.25$).

differences in mRNA levels of studied genes were found between exposed and control groups (Table III, Figures 1 and 2). mRNA levels of *TP53*, *BCL2* and *BAX* decreased with increasing concentration of styrene in blood ($R = -0.74$, $P < 0.001$; $R = -0.62$, $P < 0.001$ and $R = -0.50$, $P < 0.001$, respectively; Table III) and at workplace ($R = -0.75$, $P < 0.001$; $R = -0.65$, $P < 0.001$ and $R = -0.48$, $P < 0.001$, respectively; Table III). On the contrary, $p21^{CDKN1A}$ mRNA levels increased with increasing concentrations of styrene at workplace and in blood ($R = 0.64$, $P < 0.001$ and $R = 0.40$, $P < 0.001$, respectively; Table III). After restricting the statistical analyses to exposed subjects only, all these correlations remained statistically significant (Figures 1 and 2). Due to the strong correlation between styrene concentration at workplace and in blood ($R = 0.75$, $P < 0.001$) and the fact that styrene in blood more accurately reflects internal styrene exposure, only the latter data are shown.

Relationship between markers of DNA damage and mRNA levels of TP53, $p21^{CDKN1A}$, BCL2 and BAX

Our results show higher levels of SSBs in DNA of control individuals (1.20 ± 0.70 SSB/10^9 Da) in comparison with individuals exposed to both low (0.77 ± 0.39 SSB/10^9 Da) and high (0.51 ± 0.41 SSB/10^9 Da) styrene concentrations ($P < 0.001$; K–W test, Table IV). We found positive correlations between SSBs and EndoIII sites and mRNA levels of both *TP53* ($R = 0.389$, $P < 0.001$ and $R = 0.402$, $P < 0.001$, respectively) and *BCL2* ($R = 0.213$, $P = 0.038$ and $R = 0.358$, $P = 0.002$, respectively).

Levels of SSBs and EndoIII sites decreased with increasing $p21^{CDKN1A}$ mRNA expression levels ($R = -0.505$, $P < 0.001$ and $R = -0.309$, $P = 0.007$, respectively). No association was found between mRNA expression levels of *BAX* and markers of DNA damage.

Table IV. Parameters of DNA damage, DNA repair rates and mRNA expression levels in cell cycle genes

		All subjects	Controls	Low exposed	High exposed	*P*-value[a]
SSBs in DNA	*N*	97	37	28	32	<0.001
(SSB/10^9 Da)	Mean ± SD	0.85 ± 0.61	1.20 ± 0.70	0.77 ± 0.39	0.51 ± 0.41	
	Range	0.01–2.30	0.10–2.30	0.20–1.70	0.00–2.00	
	Median	0.65	1.22	0.73	0.44	
SSBs in	*N*	97	37	28	32	
EndoIII sites	Mean ± SD	0.46 ± 0.44	0.51 ± 0.55	0.52 ± 0.43	0.33 ± 0.27	ns
(SSB/10^9Da)	Range	0.00–2.03	0.00–2.03	0.00–1.59	0.00–1.02	
	Median	0.37	0.28	0.49	0.29	
γ-Irradiation	*N*	97	37	28	32	0.005
DNA repair rates	Mean ± SD	0.87 ± 0.91	0.65 ± 0.82	1.34 ± 1.00	0.72 ± 0.81	
(SSB/10^9 Da)	Range	0.00–3.00	0.00–3.00	0.01–3.00	0.00–3.00	
	Median	0.61	0.39	1.27	0.44	
Expression	*N*	122	51	28	43	
levels of *TP53*	Mean ± SD	$4.69 \times 10^6 \pm 3.01 \times 10^6$	$7.01 \times 10^6 \pm 3.23 \times 10^6$	$3.82 \times 10^6 \pm 1.24 \times 10^6$	$2.50 \times 10^6 \pm 0.84 \times 10^6$	<0.001
(number of	Range	1.07×10^6–16.44×10^6	2.30×10^6–16.44×10^6	1.92×10^6–7.72×10^6	1.07×10^6–5.73×10^6	
copies per 1 μg	Median	3.58×10^6	6.10×10^6	3.54×10^6	2.44×10^6	
of total RNA)						
Expression	*N*	122	51	28	43	<0.001
levels of	Mean ± SD	$1.53 \times 10^8 \pm 3.98 \times 10^7$	1.17×10^8–2.05×10^8	$1.69 \times 10^8 \pm 2.12 \times 10^7$	$1.85 \times 10^6 \pm 0.83 \times 10^6$	
$p21^{CDKN1}$	Range	63.44×10^6–2.40×10^8	63.44×10^6–2.05×10^8	$1.17 \times 10^8 \pm 2.80 \times 10^7$	1.48×10^8–2.40×10^8	
(number of	Median	1.59×10^8	1.11×10^8	1.70×10^8	1.79×10^8	
copies per 1 μg						
of total RNA)						
Expression	*N*	122	51	28	43	<0.001
levels of *BCL2*	Mean ± SD	$3.32 \times 10^6 \pm 2.11 \times 10^6$	$4.84 \times 10^6 \pm 2.26 \times 10^6$	$2.80 \times 10^6 \pm 1.19 \times 10^6$	$1.85 \times 10^6 \pm 0.83 \times 10^6$	
(number of	Range	0.58×10^6–10.26×10^6	1.05×10^6–10.26×10^6	1.58×10^6–7.50×10^6	0.58×10^6–5.04×10^6	
copies per 1 μg	Median	2.70×10^6	4.44×10^6	2.61×10^6	1.75×10^6	
of total RNA)						
Expression	*N*	121	51	28	42	<0.001
levels of *BAX*	Mean ± SD	$2.28 \times 10^6 \pm 0.53 \times 10^6$	$2.54 \times 10^6 \pm 0.51 \times 10^6$	$2.36 \times 10^6 \pm 0.42 \times 10^6$	$1.91 \times 10^6 \pm 0.39 \times 10^6$	
(number of	Range	1.14×10^6–3.67×10^6	1.78×10^6–3.67×10^6	1.47×10^6–2.98×10^6	1.14×10^6–3.19×10^6	
copies per 1 μg	Median	2.21×10^6	2.45×10^6	2.53×10^6	1.84×10^6	
of total RNA)						

Values are expressed as mean ± SD, range and median. *N*, number of individuals; SD, standard deviation; ns, not significant.
[a]Level of significance (K–W test).

Relationship between γ-irradiation-specific DNA repair rates and mRNA levels of TP53, $p21^{CDKN1A}$, BCL2 and BAX

γ-Irradiation-specific DNA repair rates were the highest among individuals with low styrene exposure (1.34 ± 1.00 SSB/10^9 Da), followed by those with high styrene exposure (0.72 ± 0.81 SSB/10^9 Da) and the controls (0.65 ± 0.82 SSB/10^9 Da).

Our results revealed a positive association between mRNA expression levels of $p21^{CDKN1A}$ and γ-irradiation-specific DNA repair ($R = 0.207$, $P = 0.044$), whereas there were no associations between mRNA levels of *BAX*, *BCL2* and *TP53* and γ-irradiation-specific DNA repair.

Discussion

In this study, we observed exposure related differences in mRNA gene expression levels of *TP53*, $p21^{CDKN1A}$, *BCL2* and *BAX* among individuals occupationally exposed to styrene. Our results may confirm the suggested close link between DNA repair and cell cycle regulation (24,25). The *TP53* transcriptional activities and its relationship to $p21^{CDKN1A}$ have been recently reviewed, and it has been shown that $p21^{CDKN1A}$ plays an essential role in DNA damage response, and regulation of this gene is complex at both mRNA and protein level (26–28). We observed lower mRNA expression of *TP53* in the exposed individuals, whereas mRNA expression of $p21^{CDKN1A}$ was lower in the control group. A similar result was detected in PBL of workers chronically exposed to benzene, where lower mRNA expression of *TP53* was observed, but no difference was found in $p21^{CDKN1A}$ mRNA levels between the exposed and control groups (8). PBL are predominantly quiescent in G_0 phase of the cell cycle. *In vitro* study on quiescent and stimulated PBL revealed that after γ-irradiation, TP53 increased only in stimulated lymphocytes, whereas $p21^{CDKN1A}$ increased in both cases and in a dose-dependent manner (29). Enhanced $p21^{CDKN1A}$ expression can occur through both TP53-dependent and TP53-independent mechanisms (30,31). When studying the effect of genotoxic agents on gene expression in cell lines, the authors observed a 3-fold increase in the synthesis of the p53 protein in TK6 cells exposed to 10 μM of diepoxybutane for 24 h, but no significant elevation of *TP53* mRNA levels was detected under the same exposure conditions (32). On the other hand, an *in vivo* assay for studying gene expression changes in epithelial cells of glandular stomach treated with *N*-nitroso-*N*-methylurea or *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine showed upregulation of $p21^{CDKN1A}$ mRNA levels in treated mice compared with controls (33). These results are in accordance with our findings of higher mRNA levels of $p21^{CDKN1A}$ in exposed individuals, but any direct comparison of the data from animal or experimental systems to humans should be confirmed by additional independent studies.

Both *BCL2* and *BAX* mRNA levels decreased with styrene exposure. This result is not in accordance with the results from the previous *in vitro* study, where overexpression of BCL2 and downregulation of BAX was observed after styrene administration (15). Harvilchuck *et al.* (34) also observed that the treatment of Clara cells with R-enantiomer of styrene-7,8-oxide or styrene resulted in increased *BAX*/*BCL2* mRNA ratio, followed by an increase in BAX/BCL2 protein ratio, depending on the time after administration. This discrepancy with our data may be due to the particular biological status of PBL.

Markers of DNA damage (SSBs and EndoIII sites) were increasing with increasing mRNA gene expression of *BCL2* and *TP53*, whereas higher DNA damage was associated with lower mRNA levels of

p21^{CDKN1A}. No association was revealed between DNA damage and mRNA levels of *BAX*. These correlations are difficult to explain in the light of nature of SSBs and EndoIII sites since they do not represent exclusively markers of DNA damage but also intermediates in the repair process (35).

In our previous study on the same population, it has been shown that γ-irradiation-specific DNA repair rates were the highest among individuals with low styrene exposure, followed by those with high styrene exposure and the controls (9). *p21*^{CDKN1A} mRNA expression levels increased with increasing γ-irradiation-specific DNA repair capacity, particularly in the control and the low exposed groups. In the high exposed group, the γ-irradiation-specific DNA repair capacity decreased in comparison with low exposed group, but the *p21*^{CDKN1A} mRNA expression continued to rise up. This phenomenon is difficult to explain. However, p21^{CDKN1A} has an active regulatory role in DNA repair. *In vitro* studies revealed the impact of p21^{CDKN1A} on BER via its interactions with proliferating cell nuclear antigen (11). Furthermore, p21^{CDKN1A} was shown to bind with poly [ADP-ribose] polymerase 1 and regulate the interaction between poly [ADP-ribose] polymerase 1 and BER factors, wherein p21^{CDKN1A} is required for the turnover of poly [ADP-ribose] polymerase 1 association with XRCC1 and DNA polymerase β to promote efficient repair (27,36). We may suggest that our finding about the relationship between DNA repair capacity and *p21*^{CDKN1A} mRNA expression level is in line with above-mentioned studies and confirms the pivotal role of p21^{CDKN1A} in the DNA repair.

Based on the above associations, occupational exposure to styrene exerts an impact on gene expression at transcriptional levels of the studied genes in PBL. So far, the existing literature does not provide any clear evidence about a possible link of the studied genes at transcriptional levels in response to DNA damage or in response to occupational exposure in humans. The relationship between styrene exposure, DNA repair and expression levels of cell cycle genes presents an interesting *in vivo* model to investigate the basic principles of cellular regulation. Our results warrant further focused studies exploring also tissue-specific and genotoxic stress-specific context.

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